Medical Lib.
APR 4 1924

THE JOURNAL

OF

EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

VOLUME XXXIX, No. 4 APRIL 1, 1924

PUBLISHED MONTHLY
AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.
BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1924, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter June 3, 1916, at the Post Office at Baltimore, Md., under the act of March 3, 1879.
Accepted for mailing at special rate of postage provided for in section 1103, act of October 3, 1917.
Authorized June 29, 1918

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. Information regarding contributions and subscriptions is given in full on the back cover.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of approximately 800 pages. The subscription price is $5.00 per volume to subscribers in the United States and Canada, payable in advance; $5.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

JACQUES LOEB, New York, N. Y. W. J. V. OSTERHOUT, Cambridge, Mass.

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. It is issued bimonthly, one volume of about 600 pages appearing in a year. The subscription price per year (one volume) is $5.00, payable in advance. Single copies cost $1.00. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of General Physiology*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

WAVERLY PRESS
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

THE PATHOGENESIS OF RHEUMATIC FEVER.

By HOMER F. SWIFT, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

PLATES 22 TO 27.

(Received for publication, June 27, 1923.)

The manifold clinical signs of rheumatic fever have been the cause of much confusion in our conception of the essential nature of the disease. Another reason for this confusion is the fact that so many diseases at times have joint pains as a part of their symptom-complex. Indeed, Wiesel (1) has recently stated that no less than 80 different pathological conditions have been included under the term rheumatism. Gradually, as more exact knowledge of various diseases has been acquired, one after another of these numerous conditions has been placed in its proper nosological position; and we now realize that inflammation of the joints or muscles is usually a symptom of some general disease. In fact, most specific bacterial infections may occasionally have arthritis as one of their complications.

But the disease known today as rheumatic fever was generally regarded, until recent years, as having arthritis as its principal manifestation. Indeed, the term acute articular rheumatism is sufficiently descriptive to indicate that historically, at least, attention was focused on the organs of locomotion. With the development of auscultatory methods and the application of statistical studies it became evident a century ago that chronic cardiac valvular disease was frequently preceded by acute arthritis (2–6). Endocarditis was then regarded as a complication or sequela of acute articular rheumatism; but we now realize that heart involvement is the most important part of the disease, and that the patient is often suffering from active visceral infection with little or no evidence of arthritis. This altered conception of the disease is reflected in changes in the names applied to it from time to time; *viz.*, acute articular rheumatism, or acute inflammatory rheumatism, acute rheumatic fever, or better

still, rheumatic fever; the last appellation is probably best because the infection not infrequently passes into a subacute or chronic form.

It is surprising that in spite of the great advance in the knowledge of the causation of most common infectious diseases we still must recognize our uncertainty of the etiologic agent in this infection. Certain contributing environmental factors have been suggested (7, 8), and non-hemolytic streptococci have been claimed by several authors (9–13) to be the causative microorganisms; but the failure to recover these streptococci from a majority of patients (14) and the fact that the classical disease presented by man has not been reproduced by inoculating them into the lower animals (15, 16) have caused most students to hesitate before considering the question settled.

To obtain a better understanding of rheumatic fever we are, therefore, forced back to a continued bedside study of the patient, with newer instrumental methods to aid our senses, and to an examination of the various tissues that can be obtained from the subject during life and post mortem. By the correlation of the different facts so obtained we should arrive at a more exact knowledge of the life history of the disease.

The gross clinical manifestations of rheumatic arthritis are pain, tenderness, swelling, redness, and local heat diffusely distributed about the joint; an examination of the synovial fluid reveals many exudative cells, mostly polymorphonuclear leucocytes. Remarkable features of the acute arthritis are (1) the tendency for the inflammation to to migrate—to jump—from one joint to another without any apparent involvement of the intervening tissues; (2) the failure of the process to go on to suppuration; and (3) the rapid disappearance of the symptoms and signs of inflammation after the patient has taken such antipyretic drugs as certain derivatives of salicylic acid or phenylcinchoninic acid.

Histopathology of Cardiac Lesions.

In fatal cases, the longest recognized (17) and most striking gross feature found post mortem is the appearance of rows of small bead-like excrescences along the free margins of the heart valves. On microscopic examination these verrucæ are seen to be made up of coagulated elements derived from the circulating blood, in other words, small globular thrombi deposited on the valvular endocardium

at a place where the lining endothelium has disappeared (18). In older verrucæ there is also definite evidence of a tendency to healing (19); the verrucæ are covered with endothelium and invaded by organizing connective tissues. But even in the young lesions there is seen in the substance of the heart valve under the endocardium distinct evidence of inflammation not exudative but proliferative in nature (Figs. 1 and 2).[1] It is a moot point whether the destruction of the endothelium is primary or subsequent to an injury to the underlying tissue. Of this, however, we are certain—that characteristic lesions occur in the mural subendocardial region without primary injury to the endothelium and, also, are found in the base of the valve leaflets (Figs. 3 and 4). It is not difficult to conceive, therefore, of the primary injury of the valves occurring in their substance rather than on their surface; and if this is true it would be better to consider rheumatic disease of these structures as a valvulitis rather than a simple endocarditis. If edema and swelling are present in the valves to the same extent as about the joints, it is easy to think of them as being functionally faulty and to see the possibility of the swollen covering endothelium being broken by repeated impacts against an opposing valve leaflet.

When the pericardium is extensively inflamed there is often a widespread pouring out of serofibrinous exudate with a plastering together of the two layers of the pericardial sac. Upon first glance this seems an entirely different process than is found in other tissues; but the presence in the pericardium of focal lesions similar to Aschoff bodies, which shortly will be described, indicates that the essential or primary pathological process is similar to that found elsewhere, but that the gross appearance is altered by the peculiar anatomical structure of the pericardial sac, and the manner in which such large endothelial membranes respond to injury.

The most generally recognized specific histological lesion of rheumatic fever is the so called Aschoff body (20, 21), which is a submiliary nodule located in the myocardium usually in close relationship with small blood vessels (Figs. 6 and 7.). There is practically always a small central area of necrosis surrounded by peculiar cells, having

[1] Clawson (Clawson, B. J., *Arch. Int. Med.*, 1924, xxxiii, 157) directs attention especially to the proliferative nature of the inflammatory process in the valves in 9 cases of rheumatic endocarditis recently studied by him.

vesicular nuclei and a cytoplasm that takes a granular red color when stained with methyl green pyronine; usually many cells are present with multiple nuclei, forming a particular type of giant cell, different from that seen in tuberculosis. Mixed with these Aschoff body cells are polymorphonuclear leucocytes and lymphocytes in various amounts, proportional to the acuteness of the general infection. Although the submiliary nodule is primarily in the interstitial tissue, the surrounding muscle fibers are often seen to be involved (Fig. 7). Indeed, certain pathologists claim that the giant cells arise from the muscle fibers, although the majority of observers think that the cells forming the nodules are derived from the endothelium of the perivascular spaces and from the endothelial lining of the blood vessels.

Changes in the blood vessels are common; not infrequently one encounters partial or complete closing of the lumina with thrombi that have probably been formed as a result of injury to the vessel wall (Fig. 5). The blood vessel may also be constricted in other ways: We have seen Aschoff bodies in the perivascular space compress one segment of the wall against another (Fig. 6). When two or more submiliary nodules are close together but on different sides of a vessel the edema, often present in the region of such foci, probably forms a constricting ring (Fig. 7). Endarteritis, with swelling and proliferation of the endothelium as well as of the other intimal cells, is not infrequently encountered in the smaller branches of the coronary arteries (Fig. 8). Interference with the circulation must lead immediately to disturbed nutrition of the muscle tissue and of the impulse-conducting fibers supplied by the involved blood vessels (22). Bedside study and electrocardiographic investigation in a series of our patients indicates that the myocardium or conduction system was disturbed in about 95 per cent of the cases (23). While it is conceivable that these functional disturbances may have been merely toxic in origin, it seems more rational to conclude that there is a direct relationship between the histopathological lesions demonstrable post mortem, and the disturbed myocardial function found during life. The transitory nature of many of these cardiac disturbances is no argument against their being due to actual focal lesions, for evidence is constantly increasing that focal lesions persist about inflamed joints, even though clinical manifestations of arthritis are present only a few days.

It is important, on the other hand, to realize that active disease of the heart may be the only demonstrable evidence of a continuing rheumatic fever infection. Recently, two fatal cases have been brought to our attention in which myocardial weakness was the sole clinical picture, and post mortem the only distinct lesions were Aschoff bodies widely disseminated throughout the heart muscle. In several patients suffering from chronic cardiac disease we have observed relapse after relapse with pyrexia and the general features of recurring infection in which all of the symptoms and signs were referable to myocardial and endocardial involvement. Post mortem, these cases have shown widespread rheumatic myocarditis, along with endocarditis and pericarditis. These correlated clinical, physiological, and pathological studies are giving us a clear conception of the chronic or relapsing nature of rheumatic fever.

Histopathology of Subcutaneous Nodules.

For many years English clinicians (24) have called attention to the frequent occurrence of fibroid nodules in the subcutaneous tissue of rheumatic children. Anatomically, they are found in the deep fascia over bony prominences and in tendon sheaths and tendons. The essential histological picture is similar to that seen in the Aschoff body (25). In close apposition to areas of cellular proliferation there is tissue destruction varying in size from small submiliary areas to long strands of hyaline necrosis affecting connective tissue fibers; combined with necrosis are deposits of fibrin (Fig. 9). Surrounding these destroyed foci are found numerous cells similar in appearance and staining reaction to the type of cells found in Aschoff bodies; multinuclear giant cells are also present (Figs. 10 and 13). In nodules it is not difficult to demonstrate these endothelioid cells arising from perivascular spaces as well as from the vascular endothelium (Figs. 11 and 12). In fact, the participation of the blood vessels in the general response is one of the most marked features of the subcutaneous nodules. Many capillaries are seen in which the swollen endothelium has practically obliterated the lumen; in the arterioles the proliferation of the endothelium at times takes the form of a crescent-shaped mass of cells, appreciably narrowing the vessel. Still other small arteries are seen obliterated by thrombi; in others the media is involved; and surrounding many of the smaller vessels there can often be seen collections of endothelioid cells evidently compressing the

walls. A participation of fibroblasts arising from the connective tissue is easy to demonstrate. A few polymorphonuclear cells and lymphocytes invade the diseased tissue and foci of edema are demonstrable. While, grossly, these nodules vary in size from 0.5 to 5 or 10 mm. it is evident upon microscopic examination that the larger nodules are composed of a conglomeration of submiliary nodules (Fig. 9). The pathological unity of the myocardial and subcutaneous lesions is, therefore, easily comprehensible.

These subcutaneous nodules attract attention clinically only on account of their mechanical presence. They are usually painless because they are not in close apposition to nerves. Involving only connective tissue which has no important function except that of a supporting structure, they are not a local source of danger. Their chief significance is that they indicate a similar process going on in such important organs as the heart or brain.

Histopathology of Joint Lesions.

The inflammation of the joints, a most outstanding feature from the patient's view-point, has been the least studied by histopathological methods. The transient nature of the arthritis, and the fact that patients rarely succumb to the acute disease easily explains the apparent gap in our knowledge. Heart failure is practically always accountable for the death of these patients at a time when the arthritis has disappeared, and hence the chief attention of the pathologist has been directed to this organ.

Nevertheless, Fahr (26) has found changes in the capsule of the knee of patients succumbing to rheumatic fever which he states are in every way comparable to the myocardial lesions. Coombs (27) makes a similar statement concerning the shoulder joint of one patient. Lately, we have examined portions of the capsule of the knee or ankle at the acute stages of rheumatic arthritis, and found focal lesions of the synovia, focal necrosis of the capsule, thrombosis of the smaller arteries, and endothelial and perivascular reactions comparable with changes found in the heart and in subcutaneous nodules (Figs. 14 to 16). The presence of many small nerves in the joint capsule and surrounding ligaments easily explains the great pain in rheumatic arthritis. The finding of distinct histological lesions in the joint capsule of a patient fully under the influence of neocinchophen, and from whom all clinical signs of arthritis had dis-

appeared, indicates that the essential rheumatic process may go on in spite of these antiphlogistic drugs.

Histopathology of Chorea Minor.

The relation of St. Vitus' dance, or chorea minor, to rheumatic fever has been discussed for many years. It has been known that valvular heart disease and chorea were frequently concomitant; also that arthritis and chorea occurred together. The relatively few studies of the brains of chorea patients reported in literature indicate that the chief lesion is vascular in origin (28). Thrombi, endothelial proliferation, and perivascular collections of round cells together with small focal changes in the nervous tissue contiguous to these vessel lesions have been described. The very small amount of connective tissue in the parenchyma of the brain, and the fact that the response of the central nervous system to injury is normally a neuroglia proliferation, would naturally cause a different histological picture in the brain than would be seen elsewhere. The finding, however, of typical Aschoff bodies in the hearts of patients dying from chorea, and the demonstration of subcutaneous fibroid nodules in others who have recovered, all support the view-point that the lesions in the various organs are all evidence of tissue response to a common causative agent—the infective agent, or virus, of rheumatic fever.

Correlation of Symptoms and Histopathological Lesions of Rheumatic Fever.

With this conception of the essential pathology of rheumatic fever, *viz.* disseminated focal submiliary nodules with edema in the contiguous tissues during the acute stages, combined with lesions of blood vessels, it is not difficult to reconcile the manifold and apparently unrelated manifestations of the disease. It is apparent that the type of response is an effort on the part of the body to limit the activity of the infective agent, a type that in many ways reminds us of the focal lesions of tuberculosis or syphilis. The edema, redness, and local heat seen about the joints with acute arthritis are evidence of an intense tissue reponse, and give the impression that exudation is the most marked feature. These gross clinical signs, however, disappear quickly, both spontaneously and following the exhibition of certain drugs. But small disseminated lesions of a focal character are

evidently present in the periarticular tissue and synovia much the same as in other organs, and are doubtless slower in undergoing complete resolution than the rapid recession of clinical symptoms would indicate. The pain and tenderness of the acute arthritis are probably due to the implication of numerous nerves in the acute exudative process. With the disappearance of extensive edema these symptoms usually disappear; but not infrequently one encounters patients in whom slight pain, stiffness, and tenderness persist in certain joints for weeks or months. These continuing symptoms might easily result from a persistence of focal lesions of a subacute or chronic character. The intensity of the response about the joints is probably an important factor in the complete healing of arthritic lesions. The synovia and perisynovial tissues are rich in blood vessels and hence are in a condition to respond quickly and intensely to numerous small focal injuries.

In subcutaneous nodules, on the other hand, the tissue involved is less vascular; acute exudation is less marked than about joints; perivascular cellular proliferation is very prominent; and the more subacute type of response is made evident by a slower disappearance of the evidence of injury. As already mentioned, the absence of nerves in the tissues implicated by subcutaneous nodules easily explains the lack of pain or tenderness about them.

With an understanding of what happens about joints and in subcutaneous nodules it is not difficult to construct a picture of the various cardiac lesions. The Aschoff body closely reproduces the changes seen about a single vessel in a subcutaneous nodule. In the heart there is a relatively smaller amount of connective tissue than is present about subcutaneous nodules, and in addition as the submiliary nodules usually occur about small arteries, their microscopic size is easily understood. Again a low degree of vascularity compared with the articular tissues explains the comparatively small amount of exudation; but transitory exudation is suggested by the rapidity with which electrocardiographic signs of myocardial involvement appear and disappear during the acute stages of rheumatic fever. Partial or complete occlusion of the arterioles would also result in a compromising of the nutrition of the portion of the heart supplied by them. There is also actual destruction of muscle fibers to explain

certain symptoms of myocardial disease. It is therefore, probably not overstressing the point to contend that in rheumatic fever disturbance of cardiac function points to the presence of focal lesions in the myocardium or cardiac blood vessels.

Focal lesions of the pericardium, if small, may result in a localized pericarditis, and, on the other hand, if widespread, may be followed by extensive exudation. In fact, outpouring of a serofibrinous exudate is the usual mode of response to extensive injury of large endothelial lined cavities like the pericardium and pleura, even though the character of lesions produced by the causative organisms in other tissues is usually focal in nature; for example, tuberculosis of the pleura or pericardium is ordinarily accompanied by a serofibrinous exudate. The organization of this exudate with the secondary changes incident to such organization is merely the logical outcome of a widespread pericarditis.

The peculiar character of rheumatic valvular endocarditis is more difficult to reconcile with the other focal lesions of this disease. As already mentioned, if we conceive of edema of the valve occurring as an exudative response about focal rheumatic lesions in the valve substance it is not difficult to see how the endothelium at the line of closure would be injured and small thrombi deposited on the valves at this site of injury. The healing of these thrombi must be necessarily accompanied by formation of new blood vessels and fibrous tissue; and with this process there is not only the scarring of the valves leading to a disturbance of their function, but also the production of a *locus minoris resistentiæ* in or about which subsequent relapses of rheumatic fever are liable to set up new foci of inflammation (Fig. 2). It is only fair to state that we are still uninformed of the exact mechanism by which chronic inflammation of the valves leads to progressive narrowing or funnel-shaped deformities.

The manner in which multiple vascular and perivascular lesions in the brain set up the symptoms of chorea is not entirely clear. In chorea, however, there is usually evidence of a widespread encephalitis. Sometimes practically all of the voluntary movements of the body are rendered incoordinate; at other times the symptoms point to a less extensive distribution of the pathological process. Nevertheless, we scarcely ever encounter evidence of large, single lesions in the central

nervous system, such as we see in syphilis. The symptoms of implication of the central nervous system by the virus of rheumatic fever, therefore, point to the existence of many small foci, probably in the corpus striatum, but also in the cerebral cortex; we may regard these foci as similar in nature to those found in other tissues of the body.

Doubtless comparable lesions in other organs or tissues are not infrequently present (26, 29), but are not detected either because they fail to give rise to symptoms or because the usual well marked manifestations of the disease outweigh them in prominence.

SUMMARY.

It is evident that there are two distinct types of response on the part of the body to the infectious agent of rheumatic fever; *viz.*, proliferative and exudative. The perivascular proliferative type of lesion, resembling an infectious granuloma, explains the subacute and chronic character of the clinical symptoms in many patients with this disease. Marked exudation of serum into the periarticular tissues and of serum and cells into the joint cavities are concomitants of the acute arthritis occurring with high fever and general intoxication; these acute exudations disappear following the administration of certain drugs. But their disappearance does not mean necessarily that all lesions of the proliferative type have resolved. In fact, we know that these last mentioned lesions, when present in the subcutaneous tissues, often continue for months; and from analogy we may conclude that they have a similar persistent character in other tissues of the body invaded by the causative agent of rheumatic fever.

BIBLIOGRAPHY.

1. Wiesel, J., *Med. Klin.*, 1923, xix, 163.
2. Pitcairn, quoted by Wells (4).
3. Dundas, D., *Med. Chir. Tr.*, 1809, i, 37.
4. Wells, W. C., *Tr. Soc. Improvement Med. and Chir. Knowledge*, 1812, iii, 373.
5. Hope, J., Treatise on the diseases of the heart and great vessels. . . . . New view of the physiology of the heart's action according to which the physical signs are explained, London, 3rd edition, 1839.
6. Bouillaud, J. B., Traité clinique du rheumatisme articulaire, Paris, 1840.
7. Newsholme, A., *Lancet*, 1895, i, 589, 657.
8. St. Lawrence, W., *J. Am. Med. Assn.*, 1922, lxxix, 2051.

9. Triboulet and Coyon, *Compt. rend. Soc. biol.*, 1898, l, 124.
10. Westphal, Wassermann, and Malkoff, *Berl. klin. Woch.*, 1899, xxxvi, 638.
11. Poynton, F. J., and Paine, A., Researches on rheumatism (collected papers), London, 1913.
12. Herry, *Bull. Acad. roy. méd. Belg.*, 1914, xxviii, series 4, 76.
13. Rosenow, E. C., *J. Infect. Dis.*, 1914, xiv, 61.
14. Swift, H. F., and Kinsella, R. A., *Arch. Int. Med.*, 1917, xix, 381.
15. Thalhimer, W., and Rothschild, M. A., *J. Exp. Med.*, 1914, xix, 417, 429. Rothschild, M. A., and Thalhimer, W., *J. Exp. Med.*, 1914, xix, 444.
16. Cecil, R. L., *J. Exp. Med.*, 1916, xxiv, 739.
17. Baillie, M., The morbid anatomy of some important parts of the human body, London, 3rd edition, 1797, 46.
18. Koeniger, H., Histologische Untersuchungen über Endokarditis, in *Arb. path. Inst. Leipzig*, 1903, No. 2.
19. Libman, E., *J. Am. Med. Assn.*, 1923, lxxx, 813.
20. Aschoff, L., *Verhandl. deutsch. path. Ges.*, 1904, viii, 46.
21. Geipel, P., *Deutsch. Arch. klin. Med.*, 1905, lxxxv, 75.
22. Parkinson, J., Gosse, A. H., and Gunson, E. B., *Quart. J. Med.*, 1919–20, xiii, 363 (extensive bibliography on disturbed rhythm of the heart in acute rheumatism).
23. Cohn, A. E., and Swift, H. F., *J. Exp. Med.*, 1924, xxxix, 1.
24. Barlow, T., and Warner, F., *Tr. Internat. Med. Cong.*, 1881, iv, 116.
25. Jacki, E., *Frankf. Z. Path.*, 1919–20, xxii, 82 (extensive review of literature on the pathology of subcutaneous nodules).
26. Fahr, T., *Virchows Arch. path. Anat.*, 1921, ccxxxii, 134.
27. Coombs, C., *J. Path. and Bact.*, 1911, xv, 489.
28. Greenfield, J. G., and Wolfsohn, J. M., *Lancet*, 1922, ii, 603 (extensive review of literature on the pathology of chorea).
29. Huzulla, T., *Verhandl. deutsch. path. Ges.*, 1914, xvii, 470.

EXPLANATION OF PLATES.

PLATE 22.

FIG. 1. Rheumatic valvular endocarditis and valvulitis. Verruca composed of thrombotic elements derived from the blood stream; there is a proliferative type of tissue response in the valve under the thrombus. × 118.

FIG. 2. Rheumatic cardiac valvulitis. Marked perivascular reaction about the vessels near the free edge of the aortic valve which was thickened because of a long standing rheumatic valvulitis. From the same heart as Fig. 1. × 312.

PLATE 23.

FIG. 3. Rheumatic carditis. Typical Aschoff bodies occurring in the subendocardial region in the wall of the left ventricle. *A*, Aschoff bodies; *E*, mural endocardium; and *M*, myocardium. × 68.

FIG. 4. Rheumatic cardiac valvulitis. Aschoff bodies found at the base of the mitral valve at a considerable distance from the myocardium. × 218.

FIG. 5. Rheumatic carditis. Small organizing thrombus occluding a blood vessel in the heart of a patient with rheumatic fever. × 112.

FIG. 6. Rheumatic myocarditis. Lumen of a small coronary blood vessel narrowed by the presence of an Aschoff body in the perivascular space. × 112.

PLATE 24.

FIG. 7. Rheumatic myocarditis. Aschoff bodies and edema compressing a branch of the coronary artery. *A*, Aschoff bodies; *E*, area of edema; *V*, compressed blood vessel; and *M*, broken and necrotic muscle fibers. × 140.

FIG. 8. Rheumatic myocarditis and endarteritis. *C*, artery with swollen intima and desquamating endothelium; and *A*, Aschoff body in the perivascular space. × 330.

PLATE 25.

FIG. 9. Subcutaneous rheumatic nodule. Multiple foci of perivascular proliferative reaction and (*S*) long strands of hyaline necrosis and deposits of fibrin. × 39.

FIG. 10. Subcutaneous rheumatic nodule showing a type of cell reaction similar to that seen in the myocardial submiliary nodule. *E*, endothelioid cells; and *G*, giant cells. × 445.

FIG. 11. Subcutaneous rheumatic nodule; marked perivascular proliferation of endothelioid cells and fibroblasts. *V*, small blood vessels with swollen lining endothelium. × 210.

PLATE 26.

FIG. 12. Subcutaneous rheumatic nodule. *S*, Swelling of the endothelium lining arterioles; and *P* small collection of endothelioid cells and a few lymphocytes in the perivascular space. × 495.

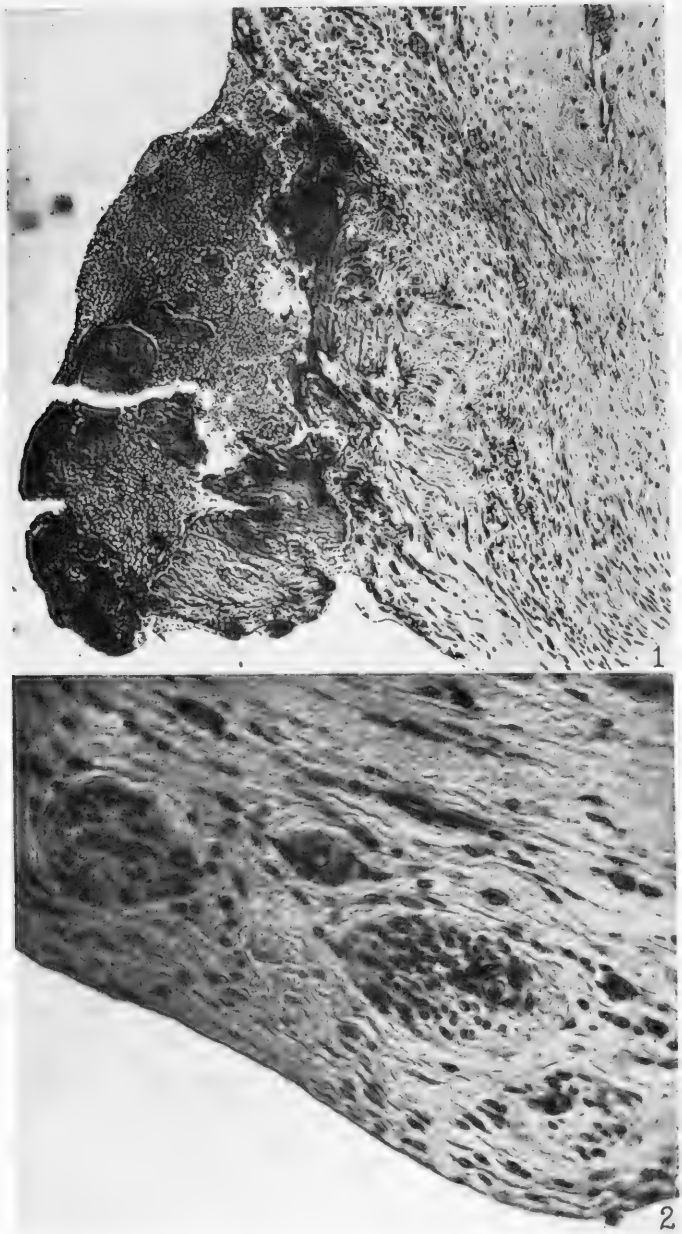
FIG. 13. Subcutaneous rheumatic nodule. *E*, endothelioid cells; *P*, polymorphonuclear leucocyte; *F*, area of focal edema; and *N*, area in which cells are undergoing degeneration. Compare with Fig. 14 showing a similar reaction in the synovia. × 234.

FIG. 14. Acute rheumatic polyarthritis. Synovia from the knee. *V*, blood vessels with swollen endothelium; *C*, swollen and actively proliferating synovia cells; and *N*, area in which cells are undergoing degeneration. × 234.

PLATE 27.

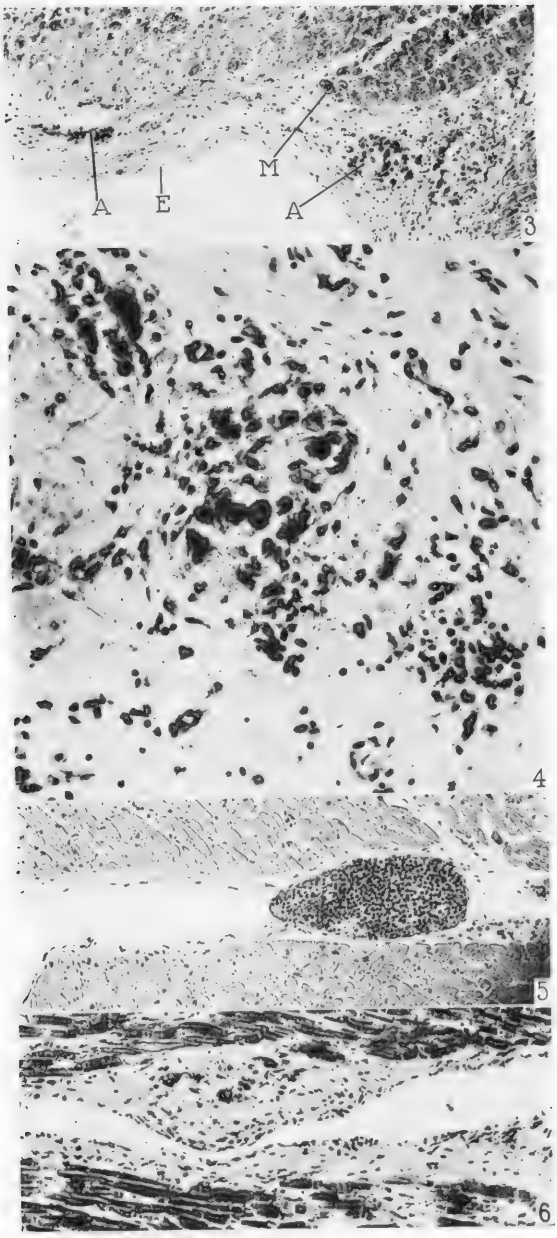
FIG. 15. Acute rheumatic polyarthritis. *N*, necrosis in the fibrous capsule of the knee with (*F*) active proliferation of fibroblasts. × 200.

FIG. 16. Acute rheumatic polyarthritis. Capsule of the knee. *E*, area of focal edema; *N*, nerve surrounded by edema; *V*, small vein with swollen endothelium; and *A*, arteriole with thickened wall compressing the lumen. × 170.



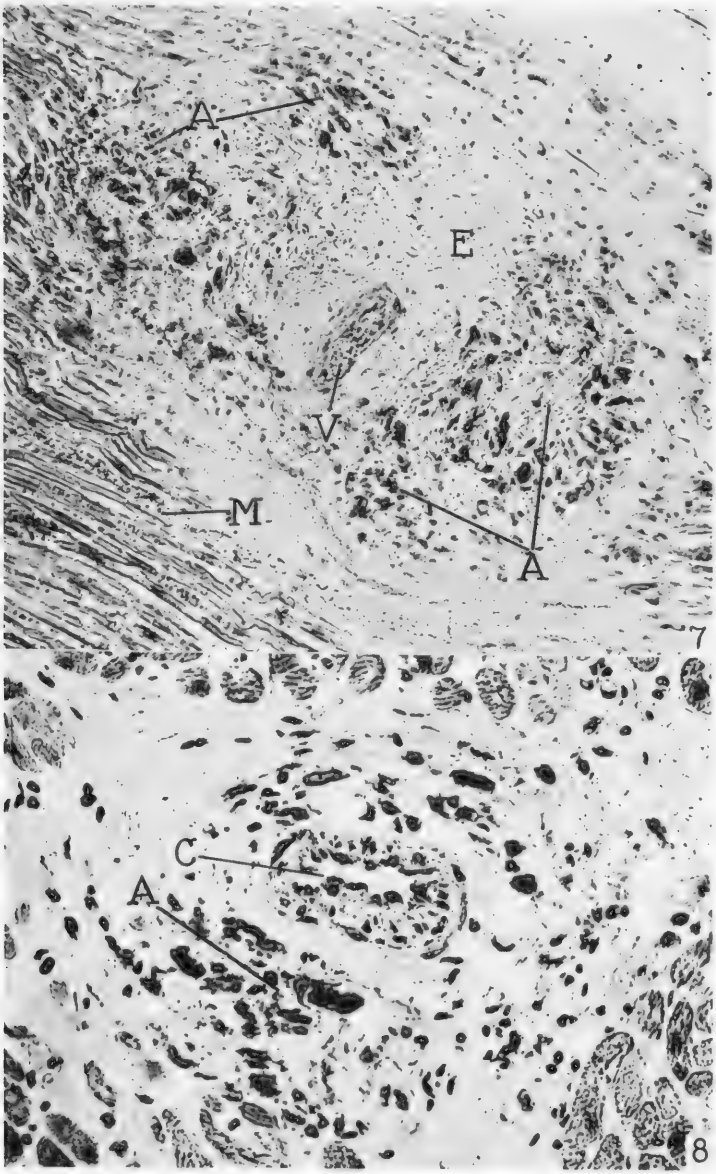
(Swift: Pathogenesis of rheumatic fever.)

UNIV. OF MICH.



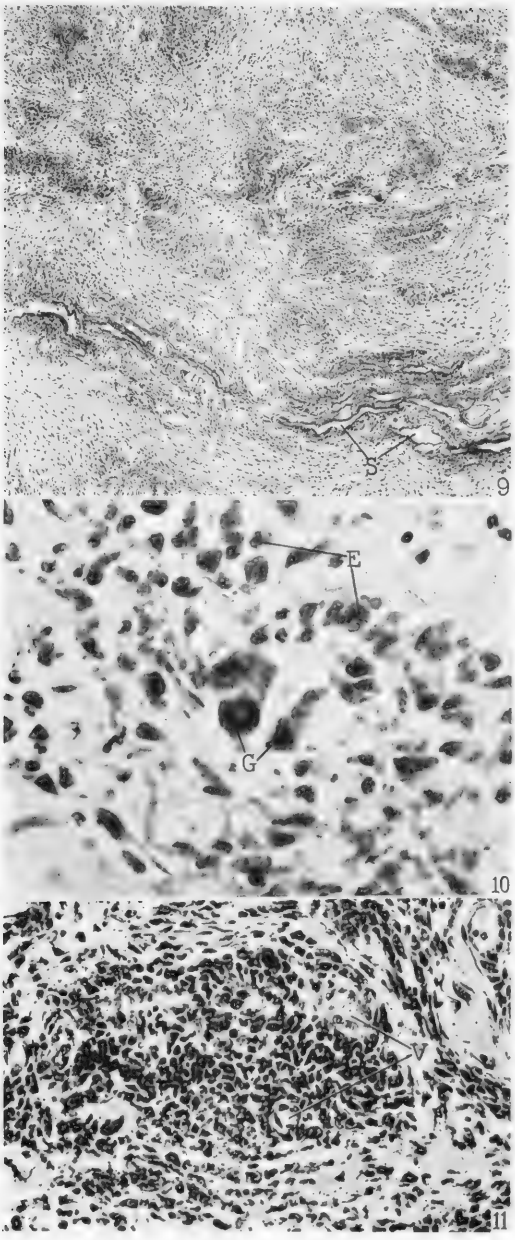
(Swift: Pathogenesis of rheumatic fever.)

UNIV. OF MICH.



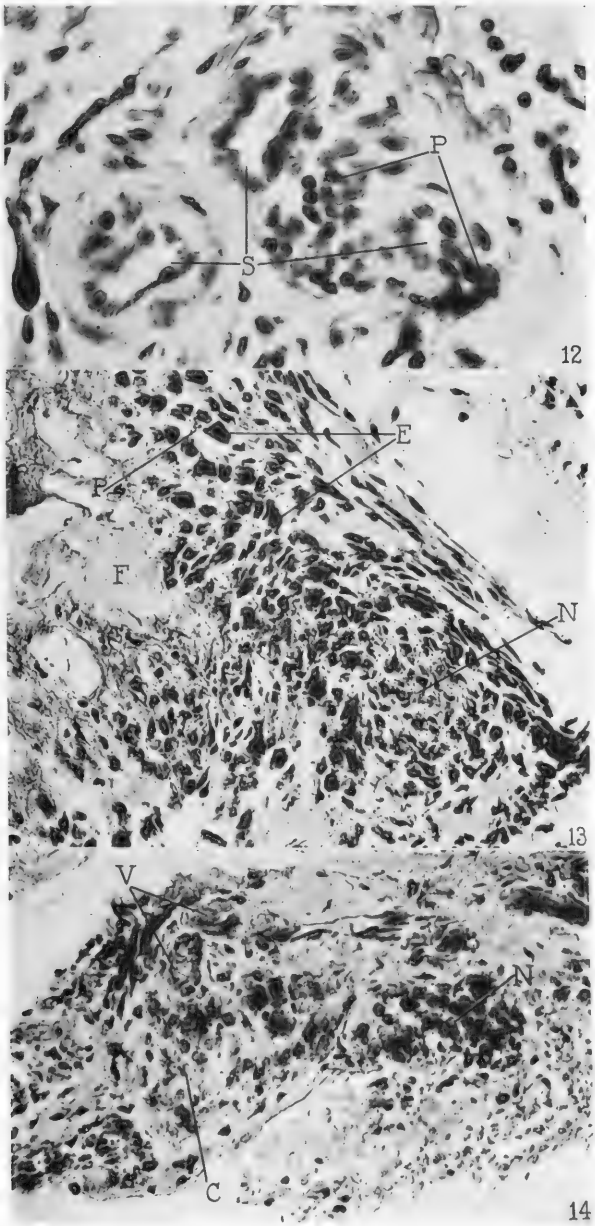
(Swift: Pathogenesis of rheumatic fever.)

UNIV. OF MICH.

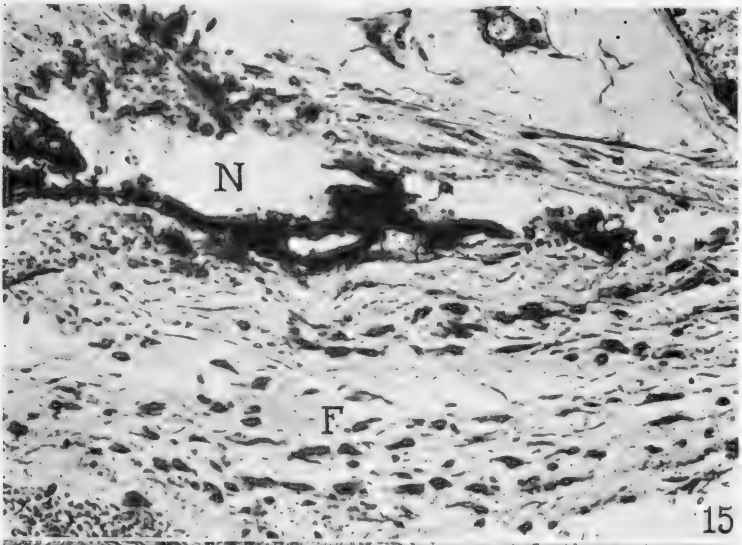


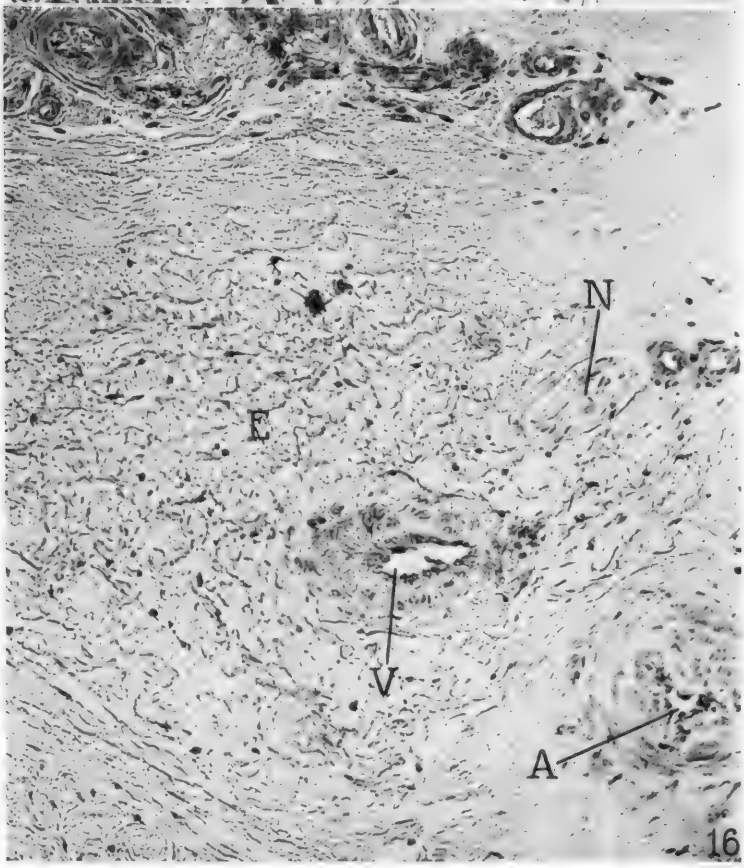
(Swift: Pathogenesis of rheumatic fever)

UNIV. OF MICH.



(Swift: Pathogenesis of rheumatic fever.)





(Swift: Pathogenesis of rheumatic fever.)

UNIV. OF MICH.

THE EFFECT OF DIGESTIVE JUICES ON THE POTENCY OF BOTULINUS TOXIN.*

By JACQUES J. BRONFENBRENNER, Ph.D., and MONROE J. SCHLESINGER, Ph.D.

(*From the Laboratory of Preventive Medicine and Hygiene of Harvard Medical School, Boston.*)

(Received for publication, September 25, 1923.)

It is a general rule that the true toxins derived from cultures of pathogenic microorganisms must be introduced parenterally if they are to act on the animal organism. When given by mouth, even in very large amounts, they tend to be harmless. The failure of the toxins to act under these circumstances is ordinarily ascribed to the fact that they are subject to destruction by the digestive juices. *Botulinus* toxin, however, presents a notable exception to the rule just mentioned. It is poisonous for experimental animals, whether administered parenterally or *per os*. Unlike other bacterial toxins, *botulinus* toxin is not usually produced within the animal body and it is doubtful whether botulism ever occurs naturally in any way other than as a result of the ingestion of a preformed toxin.

Several authors, beginning with Van Ermengem,[1,2,3] were unable to produce experimental *botulinus* infection and concluded that *B. botulinus* is a saprophyte capable of producing active toxin only outside of the animal body. Dickson[4] has confirmed these early observations with the finding that "the optimum temperature for *Bacillus botulinus* is from 24°C. to 28°C." and that "the toxin will not form at 37°C., the normal temperature of the body." Though more recently it has been shown that a majority of strains of *B. botulinus* is capable of producing the most potent toxin at body temperature in artificial media,[5] the production of toxin *in vivo* as a result of *botulinus* infection is still regarded as doubtful.

* This work was done under a grant from The National Canners' Association.

[1] Van Ermengem, E., *Z. Hyg. u. Infectionskrankh.*, 1897, xxvi, 1.

[2] Römer, P., *Centr. Bakt., 1. Abt.*, 1900, xxvii, 857.

[3] Landmann, G., *Hyg. Rundschau*, 1904, xiv, 449.

[4] Dickson, E. C., *Canad. Med. Assn. J.*, 1918, viii, 903.

[5] Orr, P. E., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 47. Dickson, E. C., and Burke, G. S., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 99.

Thus, Orr[6] in this laboratory as well as Edmondson, Giltner, and Thom[7] have found that toxin is apparently produced *in vivo* in guinea pigs inoculated or fed with toxin-free spores of *B. botulinus* but only when the quantity of spores administered is far greater than that obtaining under the circumstances of natural exposure. Their conclusion, therefore, was that, while theoretically possible, the occurrence of *botulinus* infection in nature is questionable.

In connection with the fact that *botulinus* toxin is highly poisonous when administered by mouth, the question of the exact path of absorption of the toxin naturally arises. There are several possibilities to be considered. *Botulinus* toxin may be absorbed even before it reaches the stomach; or, it may be absorbed from the stomach, and in this case it must be comparatively resistant to the action of the secretions there encountered; or again, the toxin may pass through the stomach and be absorbed by the intestinal mucosa, in which case it must not only withstand the action of the stomach juices but also the action of the secretion encountered in the intestine. Whichever set of conditions actually operates in the case of *botulinus* toxin to allow its absorption from the digestive tract, it is evident that the composition or the response to the toxin must essentially be different from that of diphtheria or tetanus toxin, since it is well known that the latter are promptly destroyed when introduced by mouth.[8, 9, 10]

The Acid-Combining Power of botulinus Toxin.

We did not attempt to determine whether *botulinus* toxin passes to any extent through the walls of the upper part of the digestive tract. It is sufficient to say that if this portion of the tract is entirely excluded, as is the case when toxin is administered through a stomach tube, intoxication proceeds at the usual rate. For this reason, our study has been limited to an inquiry into the effect

[6] Orr, P. E., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 47.

[7] Edmondson, R. B., Giltner, L. T., and Thom, C., *Arch. Int. Med.*, 1920, xxvi, 357.

[8] Ransom, F., *Deutsch. med. Woch.*, 1898, xxiv, 117.

[9] Nencki, M., Sieber, N., and Schoumow-Simanowski, E., *Centr. Bakt., 1. Abt.*, 1898, xxiii, 840, 880.

[10] Carrière, G., *Ann. Inst. Pasteur*, 1899, xiii, 435.

of changes in hydrogen ion concentration (to which the toxin is subjected in passing through the stomach and intestines) either alone or combined with the effect of the digestive ferments.

It has been stated in the literature that *botulinus* toxin is relatively resistant to acid. Thus, Landmann[11] found that it resisted exposure to N/5 HCl for 24 hours. However, neither he nor other early investigators[12, 13] took into consideration the buffer content of the vehicle fluid in which toxin is present. As can be seen from Text-fig. 1, which will be discussed more fully in succeeding papers, the acid-combining power of filtrates of *botulinus* culture is quite high, even at the beginning of the growth of the organism. But it increases to a remarkable extent as the culture ages, and coincidentally with the development and accumulation of toxin.

A flask containing about 500 gm. of chopped beef heart overlaid with 500 cc. of glucose peptone broth was richly seeded with an actively growing culture of *B. botulinus* (Type A) and incubated at 37°C. 24 hours later and every day thereafter a 10 cc. portion of clear broth was removed, filtered through a Berkefeld filter, and the filtrate thus obtained was used for determining the hydrogen ion concentration of the culture, its acid-combining power, and its toxicity for mice.

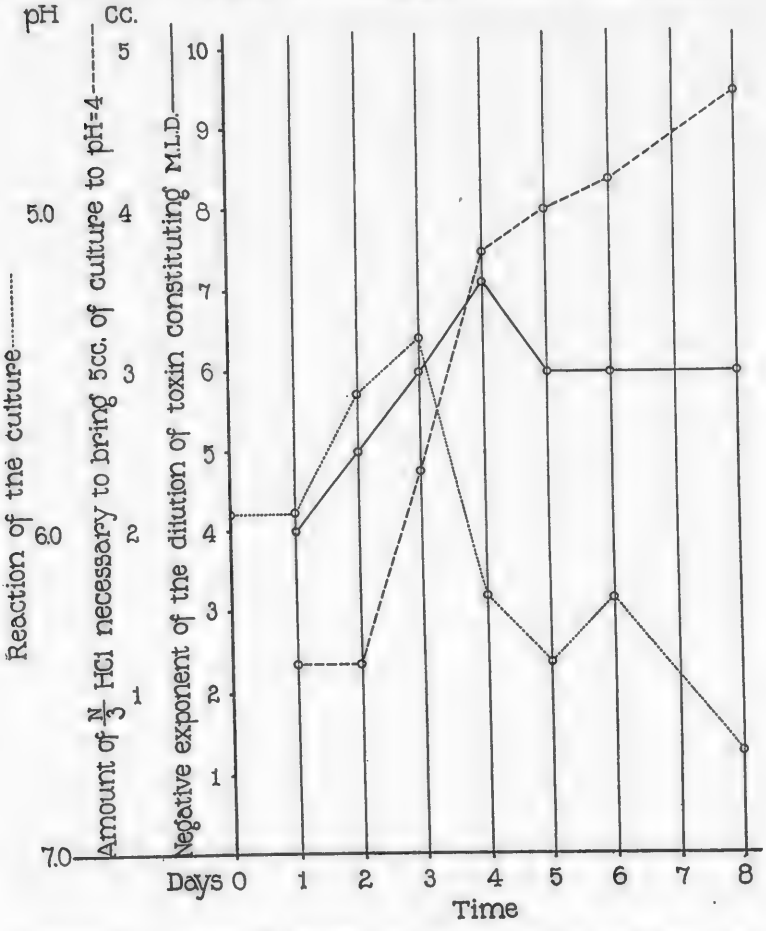
The hydrogen ion concentration was determined electrometrically by means of the potentiometer of Bovie. The acid-combining power was determined by measuring the amount of N/3 hydrochloric acid required to bring the hydrogen ion concentration of 5 cc. of the sample of broth to $C_H = 1 \times 10^{-4}$. The toxicity was determined by intraperitoneal injection into mice of serial dilutions of the filtrate, and the findings were expressed on the chart in terms of the smallest absolute amount of the filtrate causing death of mice in about 24 hours.

Resistance of botulinus Toxin to Changes in Hydrogen Ion Concentration of the Medium.—In view of wide variations in the buffer content of the culture filtrates of *Bacillus botulinus* as demonstrated above, it is evident that the early statements concerning the relative resistance of *botulinus* toxin to acid required further confirmation.

For this purpose, nine 3 cc. portions of *botulinus* toxin of known potency were mixed with equal volumes of hydrochloric acid or sodium hydroxide of different

[11] Landmann, G., *Hyg. Rundschau*, 1904, xiv, 449.
[12] Van Ermengem, E., *Z. Hyg. u. Infectionskrankh.*, 1897, xxvi, 34.
[13] Leuchs, J., *Z. Hyg. u. Infectionskrankh.*, 1910, lxv, 55.

concentrations and incubated for 24 hours at 37°C. Various concentrations of acid and alkali were obtained by mixing suitable amounts of these reagents in


pH
cc.
Reaction of the culture
Amount of N/3 HCl necessary to bring 5cc. of culture to pH=4
Negative exponent of the dilution of toxin constituting M.L.D.
5.0
6.0
7.0
Days 0 1 2 3 4 5 6 7 8
Time

TEXT-FIG. 1. Changes in hydrogen ion concentration, buffering power, and toxicity in the course of the growth of *B. botulinus*.

N/2 concentration with sufficient physiological salt solution to bring the total volume of each mixture to 3 cc. as shown in Table I. At the expiration of 24 hours of incubation, the actual hydrogen ion concentration of each toxin mixture was

determined by means of a potentiometer. Each mixture was diluted with physiological salt solution 5,000 times (making a total dilution of 10,000) and 0.3 cc. of each of these solutions was injected intraperitoneally into three mice. Thus, each one of the twenty-seven mice received 0.00003 cc. (or 3×10^{-5} cc.) of the original toxin (10 M.L.D.) modified to various degrees by incubation with acid and alkali respectively (Table I). The potency of this toxin had been previously determined on mice of 17 to 20 gm. The smallest amount killing mice by intraperitoneal injection in about 24 hours was found to be 0.000003 cc. (or 3×10^{-6} cc.).

TABLE I.

Effect of Acid and Alkali on botulinus Toxin.

Amount of toxin.	Amount of 0.5N HCl.	Amount of 0.5N NaOH.	Amount of salt solution.	Resultant pH (electrometric).		Result of injection of amount equivalent to 10 M.L.D. of toxin. Mouse. A	B	C	Period.
cc.	*cc.*	*cc.*	*cc.*						*hrs.*
3.0	3.0		0.0	2.3	Incubated for	+	+	+	6– 9
3.0	2.0		1.0	3.4	24 hrs. at	+	+	+	6– 9
3.0	1.2		1.8	4.1	37°C.	+	+	+	4– 6
3.0	0.6		2.4	5.0		+	+	+	4– 8
3.0	0.2		2.8	6.1		+	+	+	8–10
3.0			3.0	6.9		+	+	+	16–20
3.0		0.2	2.8	8.1		+	+	S.	30, 36, 96
3.0		1.1	1.9	8.9		+	S.	"	52–96
3.0		3.0	0.0	10.1		S.	"	"	96

+ indicates death; S., survival.

As will be seen from the results of this experiment, the acid did not destroy or weaken the toxin. In all cases in which toxin was incubated in acid solutions, enough remained active to kill mice; in fact, these results suggest that the toxin exposed to the action of acid killed mice more promptly than did the original toxin. For, whereas the three mice receiving the unmodified toxin died in from 16 to 20 hours, all the mice receiving the acidified toxin died in from 4 to 10 hours. The fact that the mice receiving the mixtures having an acidity equivalent to pH = 2.3 and pH = 3.4, died later than those receiving mixtures having an acidity equivalent to pH = 4.1 and pH = 5.0 may perhaps indicate that there is an optimum degree of *acidity* of the mixtures in so far as concerns their

potency. This phenomenon will be discussed more fully in one of the later papers of this series.

By contrast, the exposure of toxin to free alkali weakened it to such an extent that in the case of the mixture having a hydrogen ion concentration of pH = 8.1, one mouse out of three survived and the other two died only after 30 and 36 hours, respectively. When the hydrogen ion concentration of the mixture was still lower, the effect on the toxin was more pronounced, the mixture with a reaction of pH 8.9 killing only one out of three mice while the most alkaline mixture tested (pH 10.1) was apparently devoid of nearly all toxicity.

Digestive Action of Pepsin and Trypsin on botulinus Toxin.—Having roughly established the effect of the hydrogen ion concentration on the potency of the toxin, we next investigated the effect of the digestive ferments.

A small portion of *botulinus* toxin (about 25 cc.) was divided into two equal parts. The hydrogen ion concentration of these was adjusted to pH = 3.0 and pH = 8, respectively, by means of the addition of the proper amounts of N/2 HCl or N/2 sodium hydroxide, as determined by previous titration. The mixtures were brought to equal volume by the addition of salt solution. Two 5 cc. portions of the acidified toxin were now removed and to one of them was added 1 cc. of a 10 per cent pepsin solution, and to the other 1 cc. of salt solution. Similarly, two 5 cc. portions of the alkalinized toxin were removed and to one of them was added 1 cc. of a 10 per cent solution of trypsin, and to the other 1 cc. of salt solution. The resulting four mixtures, together with a fifth one consisting of 5 cc. of the original toxin diluted to an extent equivalent to the above mixtures, but having a hydrogen ion concentration of pH = 7.0, were incubated in test-tubes at 37°C. Small portions of the contents of the above tubes were removed and tested for toxicity by intraperitoneal injection into mice at intervals indicated in Table II.

As may be seen from the results of this experiment, the unadjusted portion of toxin, immediately after dilution, killed mice in the dose containing 3×10^{-6} cc. of the original toxin. Of the two portions of acidified toxin, the one containing pepsin retained its original toxicity even after 2 days incubation at 37°C., whereas the other portion not mixed with pepsin showed apparently an increased activity, an anomalous finding to be discussed in a later paper. Tubes 4 and 5 of the series, containing alkalinized toxin with and

without trypsin, respectively, showed gradual deterioration of the toxin as the incubation continued for days. The presence of trypsin in one of the tubes did not affect the rate of deterioration of toxin, which deterioration may for this reason be ascribed to action of the alkali alone.

TABLE II.

Effect of Pepsin and Trypsin on botulinus Toxin.

Tube No.	Amount of toxin.	Adjusted reaction of toxin.	Ferment added.	Length of time at 37°C.	Amounts of toxin injected intraperitoneally into mice (18–22 gm.) in terms of original toxin.				
					0.003 cc.	0.0003 cc.	0.00003 cc.	0.000003 cc.	0.0000003 cc.
	cc.	*pH*		*days*					
1	5.0	7.0 (unadjusted).		0	+	+	+	+	S.
2	5.0	3.0		1	+	+	+	+	+
				2	+	+	+	+	+
3	5.0	3.0	Pepsin.	1	+	+	+	+	S.
				2	+	+	+	+	"
4	5.0	8.0		1	+	+	+	S.	"
				5	+	+	S.	"	"
				10	+	S.	"	"	"
5	5.0	8.0	Trypsin.	1	+	+	+	"	"
				5	+	+	S.	"	"
				10	+	S.	"	"	"

The Ferment-Inhibiting Power of botulinus Toxin.

Since *Bacillus botulinus* exhibits marked proteolytic power, for which reason there must be associated with the toxin a certain amount of the degradation products of protein, it seemed possible that the failure of trypsin and pepsin to affect the toxin might have been due to the presence of some ferment-inhibiting substances. The titer of an arbitrary solution of trypsin was determined both in the absence of toxin and in the presence of an excess of it.

The presence of toxin did not change the titer of trypsin as determined by casein digestion, and, similarly, toxin did not interfere with the digestion of edestin by pepsin.

It is evident, therefore, that the resistance of *botulinus* toxin to the action of digestive ferments is not due to its power to inhibit the ferment activity.

SUMMARY AND CONCLUSIONS.

Botulinus toxin resists a degree of acidity equivalent to that of the stomach even when exposed thereto for 24 hours at 37°C. It is less resistant to alkali, however, as shown by the fact that in a medium of weakly alkaline reaction its potency is reduced to less than one-tenth in 24 hours. It is unaffected by peptic and tryptic digestion. Because of its instability in mildly alkaline solutions and resistance to acid, there is reason to believe that the toxin is in the main absorbed from the upper portion of the digestive tract; that is to say, from the stomach and upper duodenum.

THE EFFECT OF ANESTHESIA AND OF SEDATIVES ON THE SERUM THERAPY OF EXPERIMENTAL BOTULISM.*

By JACQUES J. BRONFENBRENNER, PH.D., AND HARRY WEISS, PH.D.

(From the Laboratory of Preventive Medicine and Hygiene of Harvard Medical School, Boston, and the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, September 25, 1923.)

The rapid progress of the intoxication in severe cases of *botulinus* poisoning in man has rendered of questionable value the therapeutic use of antitoxin in such instances.[1] The relative infrequency of outbreaks of botulism does not warrant the manufacture or distribution of this antitoxin throughout the country by commercial firms on the same scale as in the case of other therapeutic serums. As a consequence, the country is dependent upon a very few points of distribution for its *botulinus* antitoxin.[2] The delay incumbent on making the diagnosis and obtaining and administering the antitoxin usually results in the application of the antitoxin after the toxin has already produced irreparable destructive effects.

That the specific antitoxin is capable of neutralizing *botulinus* toxin has been shown repeatedly by animal experiment. We have found, however, that the time which may be permitted to elapse between the administration of toxin and the subsequent therapeutic administration of antitoxin in experimental botulism must be quite short. If delayed too long, antitoxin therapy ceases to be effective.

The length of the period that elapses after poisoning occurs, during which time antitoxin therapy continues to be effective experiment-

* The early part of this work was carried out under a grant from The National Canners' Association.

[1] Geiger, J. C., Dickson, E. C., and Meyer, K. F., *Pub. Health Bull., U.S. P.H., No. 127*, 1922, 90.

[2] These are: Hygienic Laboratory, Washington, D. C.; Laboratory of the Department of Health of the City of New York; New York State Department of Health Laboratory, Albany, N. Y.; and two or three universities where botulism is being investigated.

ally, corresponds, as we will show later, very closely to the length of time elapsing between the ingestion of the toxin and its appearance in appreciable concentration in the blood.

Since the promptness in antitoxin therapy of botulism depends directly on early diagnosis and on the availability of antitoxin, it is evident that more numerous and widespread sources of supply of antitoxin are desirable. At the same time, a procedure was sought retarding the progress of intoxication sufficiently to allow more time for the effective administration of antitoxin.

Effect of Ether on the Rate of Intoxication.—In the course of a series of experiments designed to investigate the path of absorption of *botulinus* toxin, it was necessary for the purposes of surgical manipulation to keep the animals (guinea pigs) under ether anesthesia for a prolonged period of time following the administration of large amounts of *botulinus* toxin. We observed that such animals, while under the influence of anesthesia, remained alive for hours after the death of unanesthetized guinea pigs that had received a similar amount of toxin. Because of the importance attached to any method delaying the intoxication in botulism, we investigated this apparent influence of ether anesthesia more minutely.

In these experiments, guinea pigs were given large quantities of toxin (the actual amount varying in different experiments from 15,000 to 80,000 mouse M.L.D.[3]), *per os* or intraperitoneally in order to bring about death within a few hours. Some of the animals were subjected to ether anesthesia by inhalation immediately after the administration of toxin. The administration of ether was continued for various lengths of time, usually for about 2 hours, and its effect on lengthening the life of the guinea pigs, as measured from the time of administration of toxin, was noted.[4]

[3] 1 mouse M.L.D. represents the amount of toxin causing the death of mice of 17 to 21 gm. about 48 hours after intraperitoneal administration.

[4] It was observed in the early experiments that a certain proportion of animals takes ether irregularly and in spite of all precautions in administering the anesthesia, such animals often die even during the 1st hour of the experiment. For this reason, in all the subsequent experiments only such animals were selected for the prolonged etherization as took ether evenly and remained perfectly quiet under anesthesia.

The results of four such experiments are recorded in Table I.

The Use of Anesthesia in Connection with Administration of Antitoxin.—While in the preceding experiments ether anesthesia was not continued for more than 4 hours, we had opportunity to observe, in connection with other experiments, that a more prolonged period of anesthesia resulted in a correspondingly longer period of survival of animals.

TABLE I.

The Effect of Ether Anesthesia in Delaying Death from Experimental Botulism.

Weight of guinea pigs	Dose of toxin in mouse M.L.D.	Volume of toxin.	Mode of administration of toxin.	Duration of ether anesthesia.	Result.
gm.		*cc.*		*hrs.*	
450	80,000	0.25	Intraperitoneal.		Died, 2 hrs., 5 min.
460	80,000	0.25	"		" 2 " 25 "
460	80,000	0.25	"	2	" 4 " 5 "
350	80,000	0.25	"	3	" 5 " 30 "
270	80,000	0.25	"	4	" 6 " 20 "
275	50,000	0.16	*Per os.*		" 11 "
290	50,000	0.16	" "		" 12 "
290	50,000	0.16	" "	2	" 15 " 30 min.
270	50,000	0.16	" "	4	" 18 " 10 "
350	15,000	0.075	Intraperitoneal.		" 6 "
350	15,000	0.075	"		" 6 " 20 min.
350	15,000	0.075	"	2	" 9 "
350	15,000	0.075	"	2	" 8 " 30 min.
325	20,000	0.10	*Per os.*		" 19 " 30 "
335	20,000	0.10	" "		" 18 "
335	20,000	0.10	" "	2	" 23 "
345	20,000	0.10	" "	2	" 24 " 20 min.

Table II illustrates one of these experiments.

Six guinea pigs of approximately the same weight each received 80,000 mouse M.L.D. of *botulinus* toxin, intraperitoneally. Three of the guinea pigs (Nos. 2, 4, and 4a) were immediately anesthetized (by inhalation) and kept under ether continuously for 2 hours. The other guinea pigs (Nos. 1, 3, and 3a) were left undisturbed until they began to show definite difficulty of respiration. At this time (about 1 hour after the administration of toxin) two of the guinea pigs of

each series (those under anesthesia as well as those unetherized, Nos. 3, 3a, 4, and 4a) were given intracardially a large excess of antitoxin, Animals 4 and 4a still being kept under ether for an hour more. The two remaining guinea pigs (Nos. 1 and 2) were left without antitoxin, as controls; one of them (No. 2) was still under ether anesthesia, which was continued for another hour. At the expiration of 2 hours from the time of injection of the toxin, and from the beginning of anesthesia, the administration of ether to all three guinea pigs of the first series (Nos. 2, 4, and 4a) was interrupted and the animals were left undisturbed to recover from the anesthesia.

As can be observed from the results of this experiment (Table II), the control animal (No. 1) receiving toxin alone died in 2 hours and 5 minutes. The two guinea pigs which received antitoxin as soon

TABLE II.

Effect of Ether in Rendering Effective Delayed Serum Therapy.

Guinea pig No.	Weight.	Dose of toxin (mouse M.L.D.) intraperitoneally.	Duration of anesthesia.	Time of antitoxin administration.	Result.
	gm.		*hrs.*		
1	450	80,000			Dead, 2 hrs., 5 min.
2	460	80,000	2		" 4 " 15 "
3	440	80,000		1 hr., 10 min. after toxin.	" 2 " 57 "
3a	470	80,000		1 " 5 " " "	" 2 " 50 "
4	440	80,000	2	1 " 15 " " "	Survived.
4a	450	80,000	2	1 " 20 " " "	"

as symptoms of dyspnea were observed (Nos. 3 and 3a) died very shortly after the control, thus demonstrating the inefficacy of antitoxin administered after the appearance of the first objective symptoms of poisoning. On the other hand, the guinea pig (No. 2) given the same amount of toxin but kept under ether for 2 hours immediately thereafter lived for 4 hours and 5 minutes. This delay in the case of Guinea Pigs 4 and 4a was sufficient to render serum therapy efficient[5] (Table II).

Effect of Late Administration of Ether on the Progress of botulinus Poisoning.—One of the unavoidable causes of delay in the application

[5] These two animals, as well as a number of others similarly treated, remained alive for months after the experiment.

of serum therapy in human botulism is the lack of early symptoms of poisoning. As a rule the disturbances of vision or deglutition are the first symptoms suggesting botulism. Thus, by the time *botulinus* poisoning can be suspected, a definite injury to the nervous system has already occurred. It is at this stage that serum therapy would ordinarily be attempted in a clinical case.

It is evident that a practical application of our findings (that administration of anesthesia immediately following the administration of toxin increases the likelihood that guinea pigs can be saved with antitoxin) to the therapy of botulism in human beings would be possible only if ether delays the progress of intoxication also when it is administered after the appearance of the first symptoms of poisoning.

In order to approximate more closely the conditions met with in clinical botulism, the toxin was administered to the guinea pigs of the next experiment by mouth, not by injection.

In one part of the experiment (Table III, Group 1) it was demonstrated that guinea pigs (Nos. 1, 2, 3, and 4) given 50,000 mouse M.L.D. of toxin *per os* die in from 11 to 15 hours.[6] In another part (Table III, Group 2) it was shown that among guinea pigs fed 50,000 mouse M.L.D. of toxin and given antitoxin intracardially 2, 4, 6, and 12 hours later, those receiving antitoxin during the first 4 hours after feeding of toxin may be saved (Nos. 5, 6, and 6a), while those given antitoxin later invariably died (Nos. 7, 7a, and 8).

In the last portion of the experiment (Tables III, Group 3) six guinea pigs (Nos. 9, 9a, 10, 10a, 10b, and 11), fed 50,000 mouse M.L.D. of *botulinus* toxin, were subdivided into three groups. The first group, consisting of two guinea pigs, (Nos. 9 and 9a) was put under ether and at the same time given antitoxin intracardially. The second group of three guinea pigs (Nos. 10, 10a, and 10b) received both ether and antitoxin 8 to 8½ hours after the feeding of toxin. The last guinea pig of the series (No. 11) received ether and antitoxin 10 hours after the feeding of toxin. All the guinea pigs of this series (Nos. 9, 9a, 10, 10a, 10b, and 11) were kept continuously under ether for 2 hours and at the end of this time were allowed to recover from the anesthesia. As the results of this experiment show, all the animals of this series survived except Guinea Pig 11, which was the one to receive treatment last. All the surviving animals of this series were kept under close observation for more than 2 months. On the 5th day after the completion of the

[6] As will be demonstrated in one of the later papers of this series, this irregularity in the time of death apparently depends on the amount and composition of food present in the stomach of the animals at the time of the administration of toxin.

experiment, Guinea Pig 10 was found dead. The autopsy showed intestinal stasis as a cause of death.

In view of our earlier findings, also illustrated in Table III, Group 2, that intracardial administration of antitoxin fails to save guinea pigs if given later than 4 hours after the feeding of 50,000 mouse

TABLE III.

Application of Delayed Ether Anesthesia in Conjunction with Serum Therapy of Advanced Poisoning.

Group No.	Guinea pig.		Dose of toxin fed in mouse M.L.D.	Interval between toxin and antitoxin.	Interval between toxin and ether.	Duration of anesthesia.	Result.
	No.	Weight.					
		gm.		*hrs.*	*hrs.*	*hrs.*	
1	1	270	50,000	No antitoxin.		No ether.	Died, 11 hrs.
	2	270	50,000	" "		" "	" 11 "
	3	275	50,000	" "		" "	" 12 " 50 min.
	4	275	50,000	" "		" "	" 15 " 40 "
2	5	265	50,000	2		" "	Survived.
	6	280	50,000	4		" "	Sick; survived.
	6a	245	50,000	4		" "	" "
	7	275	50,000	6		" "	Died, 17 hrs.
	7a	350	50,000	6		" "	" 17 "
	8	275	50,000	12½		" "	" 14 "
3	9	250	50,000	6	6	2	Survived.
	9a	260	50,000	6	6	2	"
	10	275	50,000	8	8	2	"*
	10a	265	50,000	8	8	2	"
	10b	295	50,000	8½	8½	2	"
	11	360	50,000	10	10	2	Died, 18 hrs.

* This animal showed no symptoms for 4 days after the completion of the experiment, but on the 5th day it was found dead. Autopsy showed that death of the animal was due to acute intestinal stasis.

M.L.D. of toxin, the survival of five guinea pigs following the application of combined ether and antitoxin therapy even up to 8 hours after the feeding of toxin is suggestive. These findings are particularly encouraging if one takes into consideration the fact that guinea pigs fed 50,000 mouse M.L.D. of toxin invariably show some symptoms of respiratory distress as early as 3 to 4 hours after the

ingestion of toxin and that 8 hours after the feeding of toxin these symptoms are very marked.

The Substitution of Luminal for Ether in the Treatment of Botulism.—In all the preceding experiments, ether was administered by inhalation. This method of administration of ether is, of course, laborious. Besides, owing to development of progressive difficulty in respiration, the administration of ether by inhalation for a period of several hours in human beings is impractical. Since the application of various other methods of administration of ether, such as the subcutaneous, colonic, etc., would have to be worked out for human beings independently of findings on animals, we limited ourselves to a few preliminary experiments in this direction. Instead, we inquired into the possibility of using substances other than ether, with the hope of obviating the difficulties connected with the administration of ether.

Acting on the suggestion of Dr. C. K. Drinker, we first investigated the usefulness of luminal sodium (sodium salt of phenylethylmalonyl urea) as a substitute for ether in conjunction with the serum therapy of botulism. According to Symes, cats can be kept under a continuous complete anesthesia by luminal for 2 and 3 weeks, under forced feeding, without any marked disturbance of metabolism.[7]

In a preliminary experiment we attempted to determine the dose of luminal sodium which could be relied upon to keep guinea pigs under deep anesthesia for a prolonged period of time, without danger to their lives. For this purpose, guinea pigs were given subcutaneously varying amounts of a 5 per cent solution of luminal sodium in sterile water after a brief (10 minutes) preliminary etherization to insure a more rapid establishment of the state of anesthesia. In this, as well as in the experiments which follow, all the animals under prolonged luminal anesthesia were kept warm artificially because of the lowered metabolism resulting from the administration of the drug.

We found that the range of usefulness of luminal sodium for guinea pigs is very limited. 50 mg. of luminal sodium per kilo of body

[7] Symes, W. L., *J. Physiol.*, 1915, xlix, p. xviii.

weight produced no visible anesthetizing effect in the guinea pig, while 150 mg. invariably killed the animals in from 36 to 48 hours. The dose of 100 mg. per kilo of body weight was found to be effective and was adopted in all the later experiments with luminal sodium.

The effect of luminal sodium anesthesia on the rate of intoxication with *botulinus* toxin and on the effectiveness of serum therapy was investigated by a method similar to that used in the study of ether anesthesia.

Having established the time of death of guinea pigs receiving 50,000 mouse M.L.D. of *botulinus* toxin *per os* (Table IV, Group 1), we next ascertained the interval of effectiveness of serum therapy (Table IV, Group 2). We found that 0.7 cc. of antitoxin, when given 2 hours after the feeding of 50,000 mouse M.L.D. of toxin, not only protected the life of guinea pigs, but also prevented the development of any symptoms of poisoning (Table IV, Group 2, Guinea Pig 5). When given 4 hours after the feeding of toxin, this excess of antitoxin protected the life of animals but did not prevent the development of initial symptoms of botulism (Table IV, Group 2, Guinea Pigs 6 and 6a). If the administration of antitoxin was delayed for 6 hours or more after the feeding of toxin, so that the animals already presented dyspnea before the injection of antitoxin, the animals invariably died (Table IV, Group 2, Guinea Pigs 7 and 7a), though their death was somewhat delayed as compared with that of controls not receiving antitoxin (Table IV, Group 1, Guinea Pigs 1 to 4). With the above data on hand, the experiment was performed as follows (Table IV, Groups 3 and 4):

A series of eight guinea pigs of approximately the same weight was divided into two groups. The first group consisted of two guinea pigs which served as controls (Table IV, Group 3). One of these (Guinea Pig 8) was given ether and while under anesthesia it received subcutaneously 100 mg. of luminal sodium per kilo of body weight. Ether was discontinued in about 10 minutes after the injection of luminal. The animal was kept in a warm room, covered, and remained under complete anesthesia for about 24 hours, after which time it gradually recovered and remained normal for 3 days. All the animals receiving luminal were treated exactly as was this control. The full description of this method will therefore be omitted and will be referred to merely as luminal anesthesia.

The second guinea pig of this group (No. 9) was fed 50,000 mouse M.L.D. of *botulinus* toxin and 4 hours later was given a subcutaneous injection (100 mg. of luminal sodium per kilo, after a brief preliminary ether anesthesia) and from this time on was treated exactly as the preceding guinea pig (No. 8). This animal died in 22 hours and 45 minutes while still under the influence of luminal, whereas the control animals died after the injection of 50,000 mouse M.L.D. of *botulinus* toxin in from 11 to 15 hours (Table IV, Group 1). This result indicated an apparently

delayed progress of intoxication under the influence of luminal. Finally, the second group of six animals of this series (Table IV, Group 4, Guinea Pigs 10 to 15) was fed, each, 50,000 M.L.D. of *botulinus* toxin and 4 hours later 100 mg. of luminal sodium per kilo of body weight were administered. From this time on, these guinea pigs were kept warm (as the controls, Nos. 8 and 9, had been). 2 hours after the administration of luminal, and 6 hours after the feeding of toxin,

TABLE IV.

The Use of Luminal Sodium in Conjunction with Serum Therapy of Experimental Botulism.

Group No.	Guinea pig.		Dose of toxin *per os*, in mouse M.L.D.	Luminal sodium (subcutaneously).			Interval between toxin and luminal.	Antitoxin.	Interval between toxin and antitoxin.	Result.
	No.	Weight.		Amount of 5 per cent solution	Amount by weight.	Dose per kilo of body weight.				
		gm.		*cc.*	*mg.*	*mg.*	*hrs.*	*cc.*	*hrs.*	
1	1	270	50,000							Died, 11 hrs.
	2	270	50,000							" 11 "
	3	275	50,000							" 12 " 15 min.
	4	275	50,000							" 15 " 40 "
2	5	265	50,000					0.7	2	No symptoms.
	6	280	50,000					0.7	4	Sick; recovered.
	6a	245	50,000					0.7	4	" "
	7	275	50,000					0.7	6	Died, 17 hrs.
	7a	350	50,000					0.7	6	" 18 "
3	8	320	None.	0.66	33	100				Anesthetized 24 hrs.
	9	320	50,000	0.64	32	100	4			Died, 22 hrs., 45 min.
4	10	320	50,000	0.64	32	100	4	0.7	6	Survived.
	11	320	50,000	0.64	32	100	4	0.7	8	"
	12	310	50,000	0.62	31	100	4	0.7	10	"
	13	320	50,000	0.64	32	100	4	0.7	12	"
	14	320	50,000	0.64	32	100	4	0.7	16	Died, 39 hrs.
	15	305	50,000	0.61	30.5	100	4	0.7	20	" 23 "

Guinea Pig 10 received 0.7 cc. of antitoxin intracardially. The remaining guinea pigs of this group received similar intracardial injections of antitoxin at intervals, as indicated in Table IV, Group 4.

The results of this and of two other similar experiments show that whereas antitoxin therapy alone failed to protect the control animals

(Table IV, Group 2, Guinea Pigs 7 and 7a) when applied 6 hours after the feeding of toxin, the guinea pigs receiving luminal could be saved by the intracardial injection of antitoxin given as late as 12 hours after the administration of toxin.

The Effect of Other Anesthetics and of Sedatives on the Rate of Toxin Absorption.—In view of recent reports of several cases of luminal poisoning among epileptics,[8] we thought it worth while to try other means of replacing ether in the serum therapy of botulism. Those substances which we tried included nitrous oxide-oxygen mixture and morphine.

Nitrous Oxide-Oxygen Mixture.—The administration of anesthesia to guinea pigs was accomplished in the following manner.

A small number of animals (from three to five) was placed in a large air-tight glass jar to which was attached a Heidbrink nitrous oxide-oxygen delivery apparatus. The mixture of gas should consist of 80 parts of nitrous oxide and 20 parts of oxygen delivered under a pressure one-fourth above atmospheric. Under these conditions, the blood receives nitrous oxide at the same rate as if the animal were breathing the pure gas at atmospheric pressure, and at the same time it receives as much oxygen as is ordinarily absorbed in normal respiration. The result is a complete nitrous oxide anesthesia without the oxygen hunger. Such anesthesia can be prolonged for hours. However, these conditions could not be maintained throughout the experiment, as guinea pigs present marked individual variation in respect to their susceptibility to nitrous oxide. It became necessary to vary both the relative proportion of gases and the pressure as dictated by the behavior of the animals in the jar. The average mixture of gas actually maintained was nearly 90 per cent nitrous oxide and 10 per cent oxygen. Pure nitrous oxide was used to induce rapid anesthesia at the beginning of the experiment. The barometric pressure in the jar was slowly raised to 100 mm. of mercury and at the end of the experiment was very slowly reduced to atmospheric. In order to test the influence of nitrous oxide anesthesia on the rate of toxin absorption and its possible utilization in serum therapy, four guinea pigs were fed 1 cc. of toxin each, and two of them were subjected to a complete anesthesia by the method described.[9]

Guinea Pigs 1 and 2 served as controls to determine the potency of the toxin. Guinea Pigs 3 and 4, receiving the same amount of toxin, were placed under anes-

[8] Phillips, J., *J. Am. Med. Assn.*, 1922, lxxviii, 1199. Grinker, J., *J. Am. Med. Assn.*, 1922, lxxix, 788.

[9] This batch of toxin was not titrated on mice, as this was considered unnecessary for the purpose of the experiment. The time of death of the controls (Guinea Pigs 1 and 2) suggests that the dose given was slightly less than 50,000 mouse M.L.D.

thesia immediately after the feeding of toxin and were kept anesthetized up to the time when both controls (Guinea Pigs 1 and 2) were dead. At this time they were allowed to recover and one of them received a large excess of antitoxin, partly intraperitoneally (3 cc.) and partly subcutaneously (2 cc.). Of these two guinea pigs, the one not having received antitoxin lived 24 hours, or several hours longer than the control animals (Guinea Pigs 1 and 2). The second guinea pig receiving antitoxin at the time both controls (Nos. 1 and 2) were dead, lived for 45 hours (Guinea Pig 4). The results are shown in Table V.

Great difficulty was experienced in these experiments owing to the varying susceptibility of the guinea pigs to nitrous oxide anesthesia. A nitrous oxide-oxygen mixture under a fixed barometric pressure that would readily anesthetize one animal would fail to affect another, and would be lethal for a third. Because of this

TABLE V.

Delay in the Rate of Intoxication Induced by Nitrous Oxide-Oxygen.

Guinea pig No.	Weight.	Amount of toxin *per os.*	Duration of anesthesia.	Time of antitoxin administration.	Result.
	gm.	*cc.*	*hrs.*		
1	263	1.05			Died, 12 hrs., 30 min.
2	305	1.02			" 17 " 10 "
3	253	1.00	17 hrs., 20 min.		" 24 "
4	245	1.00	17 " 20 "	17 hrs., 30 min. after toxin.	" 45 "

and also the prohibitive cost of the experiment, a more extended series of tests was not carried out, but the results obtained indicate that this method of anesthesia probably causes a delay in the rate of intoxication with *botulinus* toxin comparable to that observed in the case of ether or luminal sodium.

Morphine.—The most promising results of all were obtained with morphine. The fact that this drug can be given by injection (like luminal) makes it particularly suitable should this mode of therapy be attempted in human botulism.

The general procedure in studying the effect of morphine was in every respect similar to that followed with luminal sodium.

In the preliminary tests we found that the control guinea pigs receiving 50,000 mouse M.L.D. of toxin *per os* can be saved by a *subcutaneous* administration of

antitoxin, provided the latter is administered not later than 3 hours after the feeding of toxin. The guinea pig receiving 10 mg. of morphine intracardially following the feeding of 50,000 mouse M.L.D. of toxin can be saved by antitoxin administered *subcutaneously* as late as 7 hours after the feeding of toxin. One of such experiments is illustrated in Table VI.

In practically all the foregoing experiments we used an overwhelming amount of toxin (50,000 mouse M.L.D.) in order to shorten as

TABLE VI.

The Effect of Morphine in Rendering Late Serum Therapy Effective.

Guinea pig.		Toxin in mouse M.L.D.	Amount of morphine.*	Antitoxin subcutaneously.		Result.
No.	Weight.			Amount.	Interval after toxin.	
	gm.		*mg.*	*cc.*	*hrs.*	
1	290	50,000				Dead, 11 hrs.
2	295	50,000		0.5	2	Survived.
3	280	50,000		0.5	3	Dead, 47 hrs., 30 min.
4	290	50,000		0.5	5	" 10 " 35 "
5	285	50,000		0.5	7	" 11 " 30 "
6	260	50,000		0.5	9	" 11 " 25 "
7	250		10			Completely relaxed for 3 hrs.
8	285	50,000	10	0.5	3	Survived.
9	275	50,000	10	0.5	5	"
10	270	50,000	10	0.5	7	"
11	270	50,000	10	0.5	9	Dead, 71 hrs.
12	275	50,000	10	0.5	12	" 19 "

* Morphine was administered intracardially immediately after the feeding of toxin.

much as possible the period of incubation and thus facilitate the continuous observation of the animals. Before closing this investigation we thought it worth while to carry out a few complete experiments under such conditions as would reasonably approximate those encountered in clinical botulism. We assumed arbitrarily that as soon as there appeared definite symptoms making the diagnosis of botulism possible, the patient would receive the injection of morphine

by a *subcutaneous route*, while awaiting the arrival of antitoxin. It remained to determine how long the administration of antitoxin could be delayed under these conditions and still be effective. One need scarcely say that, in view of the marked difference in susceptibility of various animal species to toxin, the guinea pig experiment can at best but roughly reproduce the course of events which takes place in human botulism.

Having first found that 0.002 cc. of the lot of toxin used in this experiment usually kills a guinea pig of 250 gm. in from 72 to 96 hours, if administered by mouth, we gave each of the series of twenty guinea pigs five times this amount of toxin (0.01 cc.). Three guinea pigs were used in this series (Table VII, Group 1) to determine the time of death after ingestion of the stated amount of toxin (0.01 cc. represented by 1 cc. of a 1 per cent solution of toxin). In this, as well as in all the preceding experiments, the guinea pigs drank the toxin from the point of a measuring pipette placed at the corner of the mouth.

In order to determine how long after feeding of toxin the unsupported serum therapy is effective, a group of five guinea pigs received intracardially[10] 0.1 cc. of antitoxin, each, some time after the feeding of toxin, as indicated in Table VII, Group 2.

A third group of seven guinea pigs was treated similarly, except that it received at the time of feeding of toxin 20 mg. of morphine subcutaneously, given in a dose of 0.2 cc. of a 10 per cent solution of the drug. The interval of time elapsing between the feeding of toxin and the injection of antitoxin is indicated in Table VII, Group 3.

The fourth group, consisting of five guinea pigs, was fed toxin as the animals of the preceding group had been, but in this case morphine was administered at the time of feeding to one guinea pig only (No. 16). The remaining guinea pigs (Nos. 17 to 20) received a subcutaneous injection of 20 mg. of morphine 2, 4, 6, and 8 hours later, respectively. The last two animals of this series had definite dyspnea at the time morphine was given. All the animals remained under observation throughout the experiment, and when the three control guinea pigs which had received only the toxin (Nos. 1 to 3, Table VII, Group 1) died, the five guinea pigs of the last group (Nos. 16 to 20) were given 0.1 cc. of antitoxin each intracardially.

The results of this experiment show that if morphine is administered at the time that toxin is fed, guinea pigs can be saved by antitoxin as late as 24 to 30 hours after the feeding of a few lethal

[10] Antitoxin was given intracardially to correspond with the intravenous administration in human cases.

doses of toxin. Without morphine, the same amount of antitoxin fails to save guinea pigs even when administered as soon as 6 to 7 hours after the feeding of toxin. Moreover, if the administration of morphine is delayed 4 or occasionally even 8 hours after the ingestion of toxin, at which time the guinea pigs have already begun

TABLE VII.

How Long Administration of Morphine May Be Delayed.

Group No.	Guinea pig.		Toxin *per os.*	Morphine subcutaneously.	Interval between feeding toxin and administering antitoxin.	Result.
	No.	Weight.				
		gm.	*hrs.*	*hrs.*	*hrs.*	
1	1	230	0	None.	None.	Dead, 19 hrs.
	2	285	0	"	"	" 24 "
	3	260	0	"	"	" 23 "
2	4	245	0	"	2	Survived.
	5	250	0	"	4	"
	6	260	0	"	6	"
	7	255	0	"	7½	Dead, 49 hrs.
	8	260	0	"	12	" 41 "
3	9	275	0	0	12	Survived.
	10	280	0	0	18	"
	11	280	0	0	24	"
	12	265	0	0	24½	"
	13	300	0	0	30	"
	14	300	0	0	30*	Dead, 30–36 hrs.
	15	235	0	0	30*	" 30–36 "
4	16	235	0	0	24	Survived.
	17	235	0	2	24	"
	18	235	0	4	24	"
	19	240	0	6	24	Dead, 2 days.
	20	245	0	8	24	Survived.

Toxin dose: 0.01 cc. (1 cc. of a 1 per cent solution) *per os.*

Morphine: 0.02 cc. (in 0.2 cc. of a 10 per cent solution) subcutaneously.

Antitoxin: 0.1 cc. intracardially.

* These two animals were in a moribund condition 30 hours after the feeding of toxin, and for this reason they were given antitoxin earlier than originally planned. At this time the observer relaxed his vigilance and when the animals were re-examined 6 hours later, both were dead.

to show objective symptoms, the progress of intoxication is thereby sufficiently delayed so that serum therapy is effective as late as 24 hours after the ingestion of toxin—at a time when animals would have been dead had they not received the injection of morphine (see controls, Table VII, Group 1). In human beings, subjective symptoms precede the objective signs of intoxication, and the use of morphine would therefore be indicated at an earlier stage of intoxication.

SUMMARY AND CONCLUSIONS.

When guinea pigs are fed large amounts of *botulinus* toxin, they develop symptoms of intoxication within 6 hours and die usually within 12 hours after the feeding of toxin. If very large amounts of toxin are introduced intraperitoneally, the animals may show symptoms of intoxication at the end of the 1st hour and die usually within 2 hours following the administration of toxin.

If these animals are placed under anesthesia following the administration of toxin, the intoxication proceeds much more slowly.

Anesthesia, by ether, is effective in this manner whether the toxin is given *per os* or intraperitoneally. The life of guinea pigs kept under ether after the administration of lethal amounts of toxin is prolonged by a period approximately equal to that during which the administration of the anesthetic is continued. When anesthesia is discontinued, the intoxication proceeds at its usual rate. It appears, therefore, that administration of ether delays the rate of intoxication but does not alter the toxin or the nature of the mechanism of intoxication. Anesthesia thus delays the progress of intoxication not only when administered immediately after the intake of toxin, but when administered much later after the intoxication has already progressed far enough to cause definite objective symptoms of poisoning. At this late stage of intoxication, the unsupported serum therapy of botulism in guinea pigs usually remains without effect. If, however, the animals are anesthetized at this time and kept under the influence of ether for some time, antitoxin therapy becomes effective. Indeed the antitoxin treatment can be delayed further for several hours, provided the animal is kept under anesthesia during the interval.

Results of a similar nature were obtained with luminal sodium, nitrous oxide-oxygen mixture, and morphine used in place of ether.

In view of the fact that the published data[1] indicate that *botulinus* antitoxin has thus far failed to give beneficial results in the treatment of botulism in human beings because, as it would seem, of the rapid progress of intoxication, any method of delaying the progress of intoxication to supplement the antitoxin therapy deserves consideration.

EXPERIMENTS ON THE SURVIVAL OF THE FEBRILE HERPETIC AND ALLIED VIRUSES IN VITRO.

By JAMES E. McCARTNEY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, November 1, 1923.)

Much interest centers at present about the subject of the etiology of epidemic (lethargic) encephalitis and of febrile herpes. In both conditions a virus has been described as the incitant, but the properties of the two viruses are so nearly alike that as yet they cannot be separated by animal tests. There is a general agreement among investigators, however, regarding the qualities of the active agent, or the invisible infecting microorganism of febrile herpes associated with the experimental lesions characterizing the condition. In the case of the so called virus of encephalitis lethargica, on the other hand, different workers have isolated organisms varying widely in their morphological and cultural characteristics, each of which has been regarded as the incitant of the disease.

At the request of Dr. Flexner, I undertook to repeat certain experiments on the cultivation of the so called virus of epidemic encephalitis outside the body which were reported by Bradford, Bashford, and Wilson,[1] by Loewe and Strauss,[2] and by Thalhimer.[3] These observers claim to have isolated, by means of the Smith-Noguchi technique, organisms resembling the globoid bodies of poliomyelitis.

Although the results were negative and I was unable to confirm the work of the authors named, it seems desirable to report the experiments. Incidentally, the experimental data refute published reports of positive cultures of quite common bacterial species de-

[1] Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Quart. J. Med.*, 1918–19, xii, 88.

[2] Loewe, L., and Strauss, I., *J. Infect. Dis.*, 1920, xxvii, 250; *Proc. New York Path. Soc.*, 1920, xx, 18.

[3] Thalhimer, W., *Arch. Neurol. and Psychiat*, 1921, v, 113.

scribed, among others, by Rosenow, Towne, and Wheeler,[4] von Wiesner,[5] Brasher, Caldwell, and Coombe,[6] Stafford,[7] Morse and Crump,[8] Bastai,[9] and Ottolenghi, d'Antona, and Tonietti.[10] On the other hand, some new facts have been secured which may be of value in any further cultivation tests which may be undertaken.

EXPERIMENTAL.

Specimens of the so called encephalitic virus were obtained from Dr. Flexner. They consisted of material kindly sent him by Dr. Levaditi and the Beckley strain isolated at The Rockefeller Institute.[11] In view of the similarity of the so called virus of encephalitis to that of febrile herpes, a sample of the latter was added, the H. F. strain, propagated through many rabbit generations at The Rockefeller Institute.[11]

The three strains of virus—one from a patient suffering from epidemic encephalitis (the Levaditi sample), the second from a case of neural syphilis (the Beckley specimen), and the third from a person suffering from ferbile herpes (the H. F. strain)—comprised, then, the several materials studied. The specimens were inoculated intracerebrally into rabbits and the symptoms produced were minutely observed. As it is now well known from the many published reports,[11] these symptoms are characteristic; they show, as a rule, a quite regular evolution. Although they may be less striking at the first successful implantation of the virus, the symptom-complex becomes established when the virus is passed from rabbit to rabbit, thus permitting in most instances a diagnosis of the nature of the

[4] Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202.

[5] von Wiesner, R., R., *Wien, klin. Woch.*, 1917, xxx, 933.

[6] Brasher, C. W. J., Caldwell, J. R., and Coombe, E. J., *Brit. Med. J.*, 1919, i, 733.

[7] Stafford, C. M., *J. Lab. and Clin. Med.*, 1918–19, iv, 691.

[8] Morse, P. F., and Crump, E. S., *J. Lab. and Clin. Med.*, 1919–20, v, 275.

[9] Bastai, P., *Riv. crit. clin. med.*, 1920, xxi, 139; *Boll. Ist. Sieroterap. milanese*, 1921, ii, 197.

[10] Ottolenghi, D., d'Antona, S., and Tonietti, F., *Policlinico*, Sec. Pract., 1920, xxvii, 1075.

[11] Flexner, S., *J. Am. Med. Assn.*, 1923, lxxxi, 1688, 1785.

infection. The symptoms presented in order of appearance may be defined as fever, gnashing of teeth, muscular movements of various kinds and degrees, including convulsions, and paralyses, terminating finally in death.

The cultivation results here recorded differ from those of the investigators mentioned, and the disagreement may be ascribed to the fact that they failed to take into account what may be defined as the essentials for the establishment of the experimental disease in rabbits. In addition to the typical symptom-complex described, the action of the so called virus of encephalitis in these animals is characterized by (*a*) a definite incubation period, (*b*) absence of gross lesions in the organs, except for congestion of the brain, (*c*) absence of infection by ordinary bacteria, and (*d*) transmissability of the experimental disease from rabbit to rabbit. Cerebral lesions in this animal cannot, by themselves, or to the exclusion of these criteria, be regarded as sufficient for a diagnosis, for changes indistinguishable from those described by previous investigators as the only condition necessary for determining the experimental disease have been found in a large number of control rabbits.[11, 12]

Cultivation Tests.

In order to obtain materials especially suited to cultivation tests, the rabbits were sacrificed when *in extremis*. The brain was immediately removed with strict aseptic precautions, fragments about 1 cm. square were cut for culture, and in addition a 5 per cent emulsion was made in saline solution for intracerebral inoculation into the rabbit, as a control for the presence of the virus. At the same time, fragments of the brain were added to broth and streaked on blood agar plates, incubated aerobically and anaerobically to determine any admixture with ordinary bacteria.

Cultures in the Smith-Noguchi Medium.—The Smith-Noguchi medium was prepared in accordance with the requirements of Loewe and Strauss.[2] The ascitic fluid was free from bile, clear, and had a high specific gravity. Cultures were examined every day, and those showing contamination were discarded. Since an accurate technique was used, it was only occasionally that such accidents occurred. When clouding occurred about the tissue, such as might be indicative of growth, it happened that a similar condition was noted in the uninoculated tubes. Film preparations were made from all tubes on the 5th, 7th, 10th, and 14th days

[12] McCartney, J. E., *J. Exp. Med.*, 1924, xxxix, 51.

of incubation at 37°C. and stained by polychrome methylene blue, and by Gram's and Giemsa's methods. At the end of 10 days, subplants were made into fresh Smith-Nouguchi medium, and again 10 days later. In other words, three subcultures were made before a final result was noted.

These experiments were repeated, variations of this medium being employed, such as substitution for the ascitic fluid of rabbit serum, undiluted or diluted with Ringer's solution or with dextrose broth.

The Levaditi and Beckley strains of the so called encephalitic virus and the H. F. strain of herpetic virus yielded no macroscopic or microscopic evidence of growth in the Smith-Noguchi medium even in the third subplant.

Since we failed to isolate the microorganisms described by Bradford, Bashford, and Wilson, Loewe and Strauss, and Thalhimer, it was thought that data regarding the persistence of the viruses in this medium would indicate whether they had multiplied or had merely survived, or had died within the cultures. The viability of the viruses was tested by inoculating rabbits intracerebrally with the fluid material surrounding the tissue and with the brain fragment itself. In this way we could determine not only the presence of the viruses or their multiplication in the surrounding medium, but also the length of time they survived in the brain fragment itself.

Experiment 1.—Virus in the form of pieces of brain from a rabbit killed when *in extremis* was inoculated into a series of tubes of Smith-Noguchi medium and incubated at 37°C. Every 2nd day a tube was examined for the presence of the virus. The ascitic fluid of the Smith-Noguchi culture was poured off, centrifuged at low speed to throw down particles of brain, and the supernatant fluid retained. The fragment of the brain tissue remaining in the culture was weighed in a sterile capsule and a 10 per cent emulsion made in normal saline.

For control, similar pieces of brain from the same rabbit were placed in tubes of 10 cc. of 1 per cent dextrose veal infusion broth (pH 7.4) and incubated aerobically. The supernatant broth and the remaining brain tissue were treated in the same manner as the Smith-Noguchi medium series.

A uniform amount, 0.25 cc., was injected intracerebrally into young rabbits of similar size and weight (1,000 gm.). The tests were made several times with each of the three strains with like results. In Tables I and II are presented the details of one series of experiments (Beckley strain), the results of which are typical of those obtained equally with the Levaditi and H. F. strains.

From the foregoing protocols, the fact is evident that the so called virus of encephalitis lethargica and that of febrile herpes contained

TABLE I.

Survival of Encephalitic Virus, Strain Beckley, in Smith-Noguchi Medium.

Incubation at 37°C.	Material.	Result of rabbit inoculation.
days		
2	Centrifuged fluid.	No reaction after 21 days.
2	Brain.	Typical symptoms. Died in 5 days.
4	Centrifuged fluid.	No reaction.
4	Brain.	Typical symptoms. Died in 6 days.
6	Centrifuged fluid.	No reaction.
6	Brain.	Typical symptoms. Died in 7 days.
8	Centrifuged fluid.	No reaction.
8	Brain.	" "
10	Centrifuged fluid.	" "
10	Brain.	" "
12	Centrifuged fluid.	" "
12	Brain.	" "
14	Centrifuged fluid.	" "
14	Brain.	" "

TABLE II.

Survival of Encephalitic Virus, Strain Beckley, in Aerobic 1 Per Cent Dextrose Broth.

Incubation at 37°C.	Material.	Result of rabbit inoculation.
days		
2	Centrifuged broth.	Typical sumptoms. Died in 5 days.
2	Brain.	" " " " 5 "
4	Centrifuged broth.	" " " " 5 "
4	Brain.	" " " " 5 "
6	Centrifuged broth.	" " " " 6 "
6	Brain.	" " " " 5 "
8	Centrifuged broth.	No reaction.
8	Brain.	Typical symptoms. Died in 6 days.
10	Centrifuged broth.	No reaction.
10	Brain.	Typical symptoms. Died in 6 days.
12	Centrifuged broth.	No reaction.
12	Brain.	Typical symptoms. Died in 5 days.
14	Centrifuged broth.	No reaction.
14	Brain.	" "

in the rabbit brain and incubated in the Smith-Noguchi medium had not multiplied or even diffused into the surrounding medium after 2 days. They were, however, still viable in the brain itself up to the

6th and occasionally the 8th days, the larger the piece of brain the longer being the survival. On the other hand, when placed in dextrose broth and incubated under aerobic conditions, they were found in the surrounding fluid up to the 6th day, and 0.25 cc. of the supernatant broth produced symptoms as severe as those induced by the fresh viruses. The brain fragments in this medium were still infective on the 10th to the 12th days.

It is clear that the three samples of viruses did not multiply in the Smith-Noguchi medium. They survived in aerobic broth, in which they were demonstrated both in the supernatant fluid and in the brain fragment long after they had become inactivated in the Smith-Noguchi medium, which, therefore, should be regarded as exerting an unfavorable effect.

A further confirmation of this conclusion is afforded by a succeeding experiment in which virus was added in the form of an emulsion of ground brain tissue to the Smith-Noguchi medium and to aerobic broth, in order that a wide surface of the inoculum might be exposed to the media. Three sets of tests were made with each strain with practically uniform results.

Experiment 2.—Rabbit brains containing respectively the fresh active virus of the Levaditi, Beckley, and H. F. strains were ground up in a sterile mortar with sand. Saline solution was added to form a 20 per cent emulsion. After centrifuging at low speed to deposit the particles of brain, 1 cc. of the emulsion was added to 15 cc. of Smith-Noguchi medium, and 1 cc. to a like amount of dextrose broth.

Before incubation at 37°C., material was removed from each tube and inoculated intracerebrally into control rabbits which developed typical symptoms and died after 5 days. Fluid was withdrawn from the tubes on the 2nd and 4th days of incubation at 37°C., and the test dose, 0.25 cc., was injected intracerebrally into a series of animals.

The material from the broth medium caused typical symptoms, and death occurred as early as in the controls, whereas similar material from the Smith-Noguchi medium was entirely without effect.

This experiment shows that in the Smith-Noguchi medium at 37°C., the viruses as contained in the ground brain tissue are either killed or rendered inactive within 2 days, while in aerobic broth at 37°C., they still remain active after 4 days incubation.

Oxygen Tension in Relation to the Viability of the Viruses.—In view of the fact that under the experimental conditions the viruses survive in broth under aerobic conditions, the question arises as to the part played by oxygen tension in preserving or destroying activity.

Experiment 3.—Twelve tubes of dextrose broth were inoculated with each of the viruses in the form of pieces of fresh brain tissue, all from the same rabbit. The tubes were divided into three sets, and four tubes were incubated aerobically, four anaerobically in the Brown jar,[13] and the remainder under a petrolatum seal.[14]

On the 2nd and 4th days of incubation, the broth from two tubes of each set was centrifuged, and 0.25 cc. of supernatant clear fluid from each tube was inoculated intracerebrally into rabbits. All twelve animals thus inoculated showed typical symptoms, and death occurred within the same time as in the control animals similarly inoculated with fresh virus.

In all, three series of twelve tubes were used, each of the three viruses being employed for inoculation.

From this experiment it appears that oxygen tension as such does not play a material part in deciding either survival or destruction of the viruses.

Acidity of the Medium in Relation to the Destruction of the Viruses.—To ascertain the effect of the acidity of the medium as a factor in the destruction of the viruses, two different experiments were made. In one the Smith-Noguchi medium was buffered at pH 7.4. This is the hydrogen ion concentration of the dextrose broth in which the viruses survive. In the other was measured[15] the pH of the broth to which was added a fragment of brain. Finally, a comparison was made with the pH of the 50 per cent glycerol in which, as is known, the viruses survive for long periods of time.

Experiment 4.—Uninoculated ascitic fluid-kidney tissue medium with a petrolatum seal after 7 days incubation showed a pH of 7.2 in the upper part of the medium, and a pH of 7.1 in the fluid surrounding the kidney tissue. Smith-Noguchi medium to which brain tissue was added exhibited a pH of 7.24 at the upper and 7.2 at the lowest part of the fluid. Varying amounts of sodium phosphate buffer solution were added to this medium containing brain tissue. It

[13] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677.

[14] Gates, F. L., and Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiii, 51.

[15] The determinations of hydrogen ion concentrations were made by Dr. J. H. Northrop.

was found that two parts of ascitic fluid and one part of sodium phosphate solution (pH 8.0) added to fresh kidney and brain tissue revealed, after 7 days incubation at 37°C., a pH of 7.4 in the fluid at the bottom of the tube.

A series of tests was carried out with this buffered ascitic fluid-kidney tissue medium, with each of the viruses. In no case, on intracerebral inoculation into rabbits, was the fluid in proximity to the brain and kidney tissues active.

The pH of the dextrose broth to which a fragment of brain from a normal rabbit was added and then incubated for varying periods, compared with the pH of the glycerol, is shown in Table III.

TABLE III.

Determination of pH of Dextrose Broth and Brain, Incubated Aerobically at 37°C.

Day.	Upper layer of medium.	Lowest layer of medium.
	pH	*pH*
2nd	6.7	6.6
4th	6.22	6.22
6th	6.42	6.42

pH of 50 per cent glycerol 5.54.

It will be noted that broth in which the viruses survive is considerably more acid than the Smith-Noguchi medium in which they rapidly become inactivated; moreover, the 50 per cent glycerol in which they may survive for long periods is still more acid.

From these results it appears that the destruction of the viruses in the Smith-Noguchi medium cannot be ascribed to acid production.

DISCUSSION.

This paper is concerned with the study of the survival and multiplication of the so called virus of epidemic encephalitis and the virus of febrile herpes in artificial cultures.

Two major sets of experiments were carried out. In the first, an attempt was made to repeat the experiments reported by Bradford, Bashford, and Wilson,[1] by Loewe and Strauss,[2] and by Thalhimer.[3] These observers believe they have succeeded, by the use of the Smith-Noguchi anaerobic tissue method of cultivation, in obtaining from the so called virus of encephalitis lethargica growths of a microorganism which is visible under the microscope, and active when inoculated into rabbits.

We have been unable to confirm the published reports; on the contrary, our experiments have taught us that the Smith-Noguchi medium is not favorable even for the survival of the Levaditi and Beckley strains of the viruses, much less for their multiplication or growth. The same fact is true for the virus of febrile herpes. It has been shown, moreover, that the viruses diffusing into the fluid part of the Smith-Noguchi medium at 37°C. rapidly lose their activity; that is to say, in less than 2 days. Within the brain fragment itself, on the other hand, they remain active up to the 6th day. In no instance could any formed bodies be detected in this medium which could be regarded as microorganisms, although detritus of very fine type is always present, which might possibly be so construed.

While we found that the Smith-Noguchi medium was unfavorable to the survival of the viruses, it was ascertained that aerobic broth is less destructive to the active material. Under the experimental conditions, it was determined that the viruses diffusing into the surrounding broth remained active for 6 days, but within the brain fragment itself they survived for 12 days.

In connection with these studies an attempt was made to determine the relation of oxygen tension and of hydrogen ion concentration to the destruction of the viruses in the Smith-Noguchi medium and to their survival in aerobic broth. Changes in oxygen tension, and variations in hydrogen ion concentration between the limits of pH 7.4 and 6.2, do not exert in this relation any appreciable effect. The precise cause of the injurious action of the Smith-Noguchi medium has not been determined.

Not only do these results fail to uphold Bradford, Bashford, and Wilson, Loewe and Strauss, and Thalhimer, but they contradict also the published reports of von Wiesner, Rosenow, Towne, and Wheeler, and others, who would identify the encephalitis virus with certain common bacteria. It can be stated unequivocally that with an accurate technique the so called virus of encephalitis lethargica and the virus of febrile herpes may be passed from brain to brain in the rabbit, under conditions in which no visible microorganisms can be made to appear in films or to grow in aerobic or anaerobic culture media.

SUMMARY.

These studies fail to confirm the statements previously made that microorganisms of the class of the globoid bodies of poliomyelitis may be cultivated in the Smith-Noguchi medium from the so called virus of encephalitis lethargica. They show equally that the herpes virus does not multiply in this medium. The experiments indicate, moreover, that the medium is unfavorable to the survival of the virus, while ordinary broth under aerobic conditions is more favorable for maintaining the activity of both the encephalitic and the herpes viruses. Probably no multiplication of either takes place in the latter medium but merely a survival, and for a maximum period of 6 days in the broth itself, and 12 days in the fragment of brain tissue immersed in the broth. Finally, it has been shown that with a suitable technique the viruses can be passed from the brain of one rabbit to that of another through a long series without contamination with cocci or other common bacterial forms. Hence we regard all reports of the finding of ordinary bacteria in the brain of cases of epidemic or lethargic encephalitis as instances of mixed or secondary infection arising during life, or examples of postmortem invasion of the body, or of faulty technique at the autopsy.

STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

III. Reduction of Methylene Blue by Sterile Extracts of Pneumococcus.

By OSWALD T. AVERY, M.D., and JAMES M. NEILL, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 11, 1923.)

The two preceding papers of this series have dealt with the phenomenon of peroxide formation by pneumococcus. The first of these studies (1) concerned itself with the conditions under which this compound is formed when air is admitted to cultures of pneumococci which have been grown anaerobically. Under these conditions peroxide is formed at reactions and at temperatures inhibiting active cell multiplication. These facts suggest that the mechanism of peroxide production is dependent upon an interaction of oxygen and cell constituents, and that under experimental conditions, at least, this reaction may proceed independently of cell growth. This view is further supported by the data presented in the second paper (2) on the formation of peroxide by sterile extracts of pneumococci. The fact that soluble extracts of pneumococcus cells form peroxide when exposed to molecular oxygen is convincing evidence that this function is not dependent upon the presence of living, formed cells.

Just as the admission of air to sterile pneumococcus extracts was found to give rise to the prompt formation of peroxide, so in the present experiments it will be shown that the addition of methylene blue to these same extracts results in rapid reduction of the dye to its colorless base. The admission of air or the addition of dye to pneumococcus extracts, in the absence of living cells, induces reactions which although yielding dissimilar products, may be alike in nature, depending upon whether molecular oxygen or methylene blue serves as hydrogen acceptor.

In the following experiments, certain conditions are defined which influence the reduction of methylene blue by pneumococcus extracts; and certain similarities are pointed out which serve to relate dye reduction and peroxide formation as functions of the same or closely allied oxidation-reduction systems in the cell.

EXPERIMENTAL.

Methods.

Preparation of Extracts.—The sterile extracts of pneumococci used in the present experiments were prepared according to the methods previously described (2). They consisted either of broth extracts of unwashed pneumococci or of washed cells extracted in phosphate solution.

Technique of Methylene Blue Reduction Tests.—A stock solution of Merck's medicinal methylene blue, in concentration of 1:1,000 in water, was sterilized in the autoclave and stored in the ice box. At the time of testing, dilutions of the stock solution were freshly prepared in sterile 0.1 M phosphate solution, pH 7.5. Exhaustion of air from the test fluids was not carried out prior to the addition of the extract, but the amount of molecular oxygen present was kept uniform throughout by saturating all solutions with air at a constant temperature of 25°C., and further admission of air was prevented by sealing the tubes with vaseline. The sterile extracts of pneumococci used in the tests were treated as indicated in the protocols. The solutions containing the complete reduction system were placed in narrow tubes of uniform bore, sealed with vaseline, and incubated at 37°C.

Reduction of Methylene Blue by Sterile Broth Extracts of Unwashed Pneumococci.[1]

In repeated experiments it has been found that small amounts of the sterile cell extract possess the property of rapidly reducing large amounts of methylene blue. For example, 0.3 cc. of the sterile extract added to 1 cc. of 1:20,000 methylene blue solution effected complete reduction in 40 minutes at 37°C.; the same amount of sterile extract added to 1 cc. of 1:2,000 methylene blue (a concentration of approximately 1 mM) brings about complete reduction in 2½ hours.

Peroxide may be considered as a product resulting from the transfer of hydrogen to molecular oxygen in the same way that the leuco

[1] Unless otherwise specified, the term pneumococcus extract refers to broth extracts of unwashed cells.

base of methylene blue may be regarded as a product resulting from the transfer of hydrogen to the dye. Thus, from the nature of the processes involved in peroxide formation and methylene blue reduction, it is possible that the same system or systems in the extract may be responsible for both activities; if molecular oxygen is admitted to an active extract peroxide is formed, if methylene blue is added to the same extract, methylene white, or the leuco base of the dye, is formed. In both cases, some constituent of the extract is oxidized, with the simultaneous formation of peroxide or of methylene white, depending merely upon whether molecular oxygen or methylene blue serves as hydrogen acceptor.

With these relationships in mind, the effects of various agents on the activity of these two functions of pneumococcus extracts have been investigated in the following experiments.

Effect of Heat upon Methylene Blue Reduction by Extracts of Pneumococcus.

In order to determine the effect of heat upon the reducing action of pneumococcus extracts, the following experiments were carried out. For purposes of comparison the heat sensitiveness of the peroxide-forming and methylene blue-reducing activities of the same extract was studied under similar conditions.

1. Influence upon the Potency of the Extracts of 5 Minutes Exposure to Temperatures Varying from 55–100°C.—In the following experiment a broth extract of unwashed pneumococci was heated for 5 minutes at the stated temperatures. The ability of the heated extract to reduce methylene blue and form peroxide was then determined.

1.5 cc. portions of cell extract were placed in a series of small, agglutination tubes, sealed with vaseline, and incubated 30 minutes at 37°C. The tubes were then immersed for exactly 5 minutes in a constantly agitated water bath at the temperatures shown in the tables. After heating, each tube was cooled immediately in ice water and held under seal until all the heating tests were completed.

1 cc. portions of extract which had been heated at the different temperatures were then aerated by shaking, and further exposed to air in a thin layer at room temperature in the dark. Peroxide tests were made on the heated extract after 1 hour's exposure to air.

0.5 cc. of cell extract which had been heated at these temperatures was added to 1 cc. of a 1:2,000 methylene blue solution in M/30 PO_4. The solutions were mixed thoroughly in small, narrow tubes and then carefully sealed with vaseline. The reduction tests were incubated in a water bath at 37°C. The results are given in Table I.

It is evident (Table I) that heating pneumococcus extract for 5 minutes at 55°C. does not seriously impair either its ability to form peroxide or to reduce methylene blue. On the other hand, heating the same extract for 5 minutes at 65°C. results in practically complete loss of both of these properties, although in the case of the

TABLE I.

Comparison of Effect of Heating for 5 Minutes at Different Temperatures upon the Peroxide-Forming and Methylene Blue-Reducing Activity of Sterile Extracts of Pneumococcus.

Extract.	Peroxide formation after 1 hr. aeration.	Methylene blue reduction after 2 hrs. incubation.
Unheated control	++++*	++++†
5 min. at 55°C.	+++	+++
5 " " 65° "	−	−
5 " " 75° "	−	−
5 " " 85° "	−	−
5 " " 100° "	−	−
Broth (unheated) control	−	−

* ++++ indicates strong peroxide reaction; +++, marked peroxide reaction.

† ++++ indicates complete reduction; +++, almost complete reduction.

methylene blue reaction, delayed and slight reduction was evident after 72 hours.

Extracts heated for 5 minutes at or above 75°C. failed to show any evidence of reducing action even after 3 days. These results indicate that the methylene blue-reducing and peroxide-forming powers of pneumococcus extracts are both susceptible to the destructive action of heat and that each of these activities shows the same rapid increase in rate of destruction when an extract is exposed to temperatures between 55° and 65°C.

2. Effect of Heating at 55°C. upon the Methylene Blue-Reducing Power of Pneumococcus Extracts.—In the following experiment the

rate of destruction of the reducing power is followed by comparisons of the final amount of methylene blue reduced by an extract which had been previously heated for periods of from 5 to 60 minutes at 55°C.

0.5 cc. of the sterile cell extract was placed in a number of narrow tubes, sealed with vaseline, and incubated at 37°C. for 40 minutes. These tubes were then heated anaerobically for different periods at 55°C. in a constantly agitated water bath. Each tube was cooled at once after the heating period and kept under seal until all tubes had been heated.

The concentrations of methylene blue used were 1:3,000, 1:7,500, and 1:15,000. These solutions were brought to constant oxygen tension by saturation with air at

TABLE II.

Influence of Heating Sterile Pneumococcus Extract upon the Total Amount of Methylene Blue Reduced at Final Equilibrium.

Cell extract heated at 55°C. (0.1 cc.).	Reduction of methylene blue (1.4 cc.).		
	1:3,000	1:7,500	1:15,000
Unheated.....	+++*	++++	++++
Heated 5 min.....	++	++++	++++
" 10 "	−	++	++++
" 30 "	−	−	++
" 60 "	−	−	+
Broth control.....	−	−	−
PO_4 solution, control....	−	−	−

* ++++ indicates complete reduction; ++, partial reduction; +, slight reduction; and −, indistinguishable from control.

25°C. 0.1 cc. of heated extract was then added to 1.4 cc. of each concentration of methylene blue solution. As controls, 0.1 cc. of broth, or 0.1 cc. of phosphate solution, was added to each dilution of the dye. All of the reduction tests were sealed with vaseline, and incubated at 37°C.

After 72 hours incubation, the reduction of the dye was determined by comparison with the respective controls.

The data in Table II show that the longer a pneumococcus extract is exposed to 55°C. the less is the amount of dye reduced by equivalent amounts of extract. The gradual decrease in the dye-reducing power of the heated extract may be considered as due to a gradual destruction of the total oxidizing-reducing activity of the

extract. In an analogous experiment in the preceding paper (2), heating an extract at 55°C. was found to effect a gradual decrease in its power to form peroxide. It is evident, therefore, that heating pneumococcus extract for 1 hour at 55°C. results in a gradual loss, but not total destruction, of both its peroxide-forming and methylene blue-reducing properties.

Activation of Sterile Extracts of Washed Pneumococci.

In the preceding paper (2) it was pointed out that thorough washing of pneumococci before extraction deprived the extracts of their capacity to form peroxide on exposure to oxygen. It was further shown that these inactive extracts acquire this property on the addition of the cell washings, muscle infusion, or yeast extract. In the following experiments methylene blue was added to washed cell extracts to determine whether they still retained their reducing action; or if this activity were lost, whether it could be restored again by adding substances known to reactivate the peroxide-forming function of these same extracts.

0.6 cc. of unfiltered, sterile extract of washed pneumococci was added to an equal volume of either muscle infusion or yeast extract which had been previously adjusted to pH 7.5. The test solutions were all brought to uniform oxygen tension by saturation with air at 25°C. To this mixture 0.1 cc. of 1:2,000 solution of methylene blue was added. The small tubes containing the test system were sealed with vaseline and incubated at 37°C. As controls double quantities of washed cell extract alone, and 0.6 cc. quantities of yeast extract or muscle infusion without pneumococcus extract, were added to 0.1 cc. amounts of methylene blue. The total volume was kept constant by the addition of sterile phosphate whenever necessary. The results of this experiment are shown in Table III.

Table III presents evidence that sterile extracts of washed pneumococci are unable by themselves to bring about the reduction of methylene blue. It is further demonstrated in this experiment that washed cell extracts in which reducing activity has ceased, may be stimulated to carry on reduction processes by the addition of muscle infusion and yeast extract.[2] The presence of these activating sub-

[2] Muscle infusion: The infusion was prepared by the routine procedure followed in the laboratory in the preparation of meat infusion broth. 500 gm. of lean beef were allowed to infuse in 1 liter of distilled water overnight at ice box temperature.

stances, which do not themselves reduce the dye, restores to washed cell extracts the power to decolorize methylene blue. This complementing action is wholly analogous to the activation by these same substances of the peroxide-forming power of extracts of washed pneumococci. Harden and Zilva (3) have found that saline suspensions of washed living colon bacilli are by themselves unable to reduce methylene blue, but exhibit this property upon the addition of various substances, among others cell washings, broth, glucose, and peptone.

Influence of Exposure to Air.—When a broth extract of unwashed pneumococci is exposed to air, it suffers a rapid loss of its ability to decolorize methylene blue; if the exposure to oxygen is continued

TABLE III.

Activation of Reducing Activity in Sterile Extracts of Washed Pneumococci.

Sterile extract of washed pneumococci.	Activating substance.		Sterile phosphate solution pH 7.5.	Methylene blue 1:2,000.	Result.	
	Muscle infusion.	Yeast extract.			Reduction.	Time.
cc.	*cc.*	*cc.*	*cc.*	*cc.*		
1.2				0.1	Negative.	5 days.
0.6	0.6			0.1	Complete.	2½ hrs.
	0.6		0.6	0.1	Trace.	5 days.
0.6		0.6		0.1	Complete.	6 hrs.
		0.6	0.6	0.1	Trace.	5 days.

for 12 to 24 hours the methylene blue-reducing action of the extract is completely destroyed. Reference has already been made to the fact that extracts prepared from washed cells are unable to induce reduction of methylene blue in the absence of certain activating substances. Extracts of this nature may be exposed to molecular oxygen for considerable periods with relatively little effect on their

After filtration through coarse paper, the infusion was sterilized for 20 minutes at 100°C. on 3 successive days. The reaction was adjusted to pH 7.5 before use.

Yeast extract: 100 gm. of brewer's yeast in 400 cc. of distilled water were adjusted to pH 4.5 and boiled for 10 minutes. The cellular material was allowed to sediment at room temperature and the clear supernatant was pipetted off and tested for sterility. The extract was stored in the ice box and the reaction adjusted to pH 7.5 before use.

reducing action when subsequently activated by the addition of these complementing substances. If, on the other hand, these same extracts are first activated and then exposed to air, they show a progressive decrease in dye-reducing activity, although the loss is not so pronounced as that which occurs on exposure of the unwashed cell extracts.

The inability of previously oxidized extracts to cause reduction of the dye may be attributable to the loss in activity resulting from the irreversible oxidation of certain constituents of the extract. The loss of this power may also be due in part to the deleterious action on the reducing system, of the peroxide which accumulates in the extract during exposure to air.

Consumption of Molecular Oxygen by Sterile Extracts of Pneumococcus.

From the data in this paper it is evident that sterile extracts of pneumococci cause complete reduction of methylene blue in solutions previously saturated with air. This fact indicates that these extracts are able to consume dissolved oxygen in the presence of methylene blue. However, the production of peroxide by these extracts upon exposure to air is evidence of their ability to consume or accept molecular oxygen in the absence as well as in the presence of methylene blue. Quantitative analyses of oxygen consumption are now being made in a study of this function of the extracts.

DISCUSSION.

In the present work, as in the experiments recorded in the preceding paper, the sterile extracts of pneumococcus employed have been of two types: one type represents broth extracts of unwashed pneumococci; the other, extracts of washed pneumococci which have been extracted in phosphate solution. These two types of extract exhibit marked differences in behavior. The extract prepared from unwashed cells extracted in broth contains a complete oxidizing and reducing system, capable in itself of forming peroxide, or of reducing methylene blue upon the admission of molecular oxygen or the addition of the dye. The second type of extract, prepared from the washed

bacteria cannot by itself initiate either of these reactions without the addition of certain substances found in meat infusion and yeast extract. The presence of these complementary substances "completes" the potentially active system or systems in the washed cell extract and renders it reactive both with molecular oxygen and with methylene blue.

Both types of pneumococcus extract are thermolabile and lose their activity on exposure to 65°C. However, the complementing substances in meat infusion and yeast extract which serve to complete the unheated extracts of washed cells are thermostable. These facts indicate that the active peroxide-forming and methylene blue-reducing systems in pneumococcus extracts consist of two component parts, one sensitive to heat, and the other relatively heat-resistant. Further analysis indicates that the thermolabile component is derived from the bacterial cell and may be ferment-like in nature. The thermostable component can be artificially supplied from sources other than those of bacterial origin. Neither of these components by itself is reactive with molecular oxygen or methylene blue, each being separately inactive and both being essential for the formation of peroxide and the active reduction of the dye. The union of these extract constituents provides an active and complete system in which or by which some substance is oxidized with the formation of peroxide or the reduction of methylene blue, depending upon whether molecular oxygen or the dye functions as hydrogen acceptor. The complex nature of the extracts themselves and especially the unknown character of the substances actually oxidized in the extracts, impose certain limitations on the interpretation and analysis of the phenomena.

In the bacterial extracts employed in these studies, the systems responsible for peroxide production and methylene blue reduction have so many characteristics in common that it seems probable that they are either identical or closely related.

SUMMARY.

1. Sterile broth extracts of unwashed pneumococci, entirely free from living or intact cells, actively reduce methylene blue.

2. Sterile extracts prepared by extracting washed pneumococci in phosphate solution are unable by themselves to reduce methylene

blue. Upon the addition of meat infusion or yeast extract, these washed cell extracts actively reduce methylene blue.

3. The system or systems in pneumococcus extracts responsible for methylene blue reduction are destroyed by exposure to temperatures practically identical with those which have been previously found to destroy the peroxide-forming activity of the same extracts.

4. It is suggested that peroxide formation and methylene blue reduction by pneumococcus extracts are functions of the same or closely related systems, the particular reaction induced depending upon whether molecular oxygen or methylene blue serves as hydrogen acceptor or oxygen donator.

BIBLIOGRAPHY.

1. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 347.
2. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
3. Harden, A., and Zilva, S. S., *Biochem. J.*, 1915, ix, 379.

EXPERIMENTAL OBSERVATIONS ON THE "CURE" OF SYPHILIS IN THE RABBIT WITH ARSPHENAMINE.*

By ALAN M. CHESNEY, M.D., and JAROLD E. KEMP, M.D.

(*From the Syphilis Division of the Department of Medicine of Johns Hopkins University, Baltimore.*)

(Received for publication, October 31, 1923.)

HISTORICAL.

The first therapeutic studies of arsphenamine were carried out by Ehrlich and Hata (1) on mice and rats infected with the organism of relapsing fever, on fowls with fowl spirillosis, and on rabbits with experimental syphilis. Rabbits with scrotal chancres were treated with the drug and the rate of healing of the lesion and of the disappearance of treponemata was noted. Rapid disappearance of the clinical phenomena and of the treponemata was interpreted by Ehrlich and Hata to mean that the animals had been sterilized, in other words, that complete biological cure had been obtained. The more recent knowledge of the behavior of syphilis in the rabbit permits us to say that these criteria of cure are altogether insufficient.

Kuznitsky (2), working under Neisser's direction, studied the effects of arsphenamine upon syphilis in apes and obtained what he regarded as certain cure in six out of nine animals treated. The criterion for cure adopted by this worker was the success or failure of reinoculation. If reinoculation proved to be successful the animal was regarded as having been rid of all its spirochetes; if unsuccessful, it was thought that infection still existed. In therapeutic experiments with other arsenicals and with various mercurials Neisser (3) had used either the reinoculation method as a criterion of cure, or had inoculated emulsions of internal organs (liver, spleen, bone marrow) of treated animals into normal apes to determine whether

* Aided by a grant from the United States Inter-Departmental Social Hygiene Board.

or not the treated animals still harbored virulent treponemata. He does not seem to have used the two methods simultaneously in connection with the same animal, but either method was considered sufficient for determining whether or not cure had been effected. Apparently the same degree of importance was attached by Neisser to each method.

Space does not permit a discussion of the data upon which Neisser concluded that the reinoculation method offers a proper criterion of cure and hence is a satisfactory method for the evaluation of an antisyphilitic agent. Uhlenhuth and Mulzer (4) came to the conclusion that in rabbits the reinoculation method was not a satisfactory one for determining whether or not syphilis could be cured. These workers recognize the discrepancy between their results and those of Neisser and suggest as an explanation that the behavior of rabbits may be different from that of apes.

In spite of these observations of Uhlenhuth and Mulzer the reinoculation method has been accepted as a satisfactory criterion of cure by Kolle (5), who concluded on the basis of this method that with the modifications of arsphenamine (novasurol, silverarsphenamine, neoarsphenamine) with which he worked, a biologic cure of syphilis in the rabbit is impossible if the treatment be delayed until as late as 90 days after infection, but is possible if treatment be begun 45 days after infection. Frei (6) also adopted the reinoculation method in studying the efficacy of silverarsphenamine and neoarsphenamine, and obtained results confirming in general those of Kolle. However, he raises the question of the validity of the reinoculation method as a criterion of cure, and suggests that it should be checked up by the tissue transfer method. It should be stated that his work appeared several months after the experiments quoted in this paper were begun.

The demonstration by Pearce and Brown (7) that in experimental syphilis in the rabbit the infecting microorganism exhibits a constant tendency to localize in the lymphoid tissues of the host and remain viable there for long periods of time, pointed the way to the possibility of using these tissues as test materials for the study of the efficacy of antisyphilitic agents. While Neisser was the first to study the results of transfer of the internal organs of infected animals

in the evaluation of antisyphilitic agents, his method involved the sacrifice of the animal and thus entailed obvious disadvantages. By means of lymph node transfer method Pearce and Brown (7) showed that a single dose of arsphenamine of 6 mg. per kilo of body weight or of neoarsphenamine of 9 mg. per kilo was insufficient to eliminate all the virulent treponemata from the popliteal lymph nodes of infected rabbits.

Since the appearance of their work the method of lymph node transfer for the evaluation of spirillicidal agents has been used by Hill and Young (8) in their studies of synthetic compounds of mercury, and recently Nichols and Walker (9) have made use of it in studying the effectiveness of arsphenamine and neoarsphenamine in experimental syphilis in the rabbit. The latter found that the popliteal nodes of rabbits infected with *Treponema pallidum* and treated intravenously with one to four doses of arsphenamine (10 mg. per kilo) or of neoarsphenamine (15 mg. per kilo) would not transmit the infection to normal rabbits. Treatment was carried out from 110 to 174 days after infection, at a time when the more acute manifestations of the disease had subsided, and node transfer was accomplished from 28 to 61 days after treatment was terminated. These experiments offer convincing proof of the efficacy of these two drugs in eliminating virulent treponemata from the lymphoid tissues of the experimentally infected animal. Voegtlin, Armstrong, and Dyer (10) were able to abolish lymph node infection in the rabbit by single doses of sulfarsphenamine and neoarsphenamine.

The Problem.

From the foregoing resumé of the literature it would appear that there is no unanimity as to the possibility of curing syphilis in the rabbit by the aid of arsphenamine or its derivatives. Judged by the experiments in which the reinoculation method is used as a test it would seem impossible to eradicate the infection once it has been well established. On the other hand, if the method of lymph node transfer be employed it would seem possible to cure this infection in the rabbit. It is furthermore apparent that there is no unanimity on the question of whether or not a successful reinoculation of a rabbit with syphilitic virus indicates that the animal has been rid of a preexisting syphilitic infection.

In view of the recently renewed interest in the chemotherapy of syphilis, as evidenced by the search for more effective mercurials and the introduction of modifications of arsphenamine, it seemed advisable to attempt to ascertain, if possible, how far the method of lymph node transfer checks up with the method of reinoculation as a criterion of cure, and to what extent arsphenamine is of value in the treatment of the experimental disease using these two methods simultaneously as criteria. Stated in other words, is it possible by the administration of arsphenamine to render syphilitic rabbits free of treponemata as judged by lymph node transfer, and are such treponema-free rabbits susceptible to a second inoculation with the same strain? The experiments herein reported constitute an attempt to answer that question. We have limited ourselves to a study of arsphenamine alone, because of the general feeling among syphilologists, in this country at least, that this drug is still superior to any of the modifications thus far produced.

Technique.

For the purposes of the experiment twenty-two male rabbits were employed. These were of varying breeds, grays and browns predominating. They were inoculated in a variety of ways with testicular emulsion containing *Treponema pallidum* (Nichols strain). Some were inoculated intradermally on the sheath of the penis, some intratesticularly (only one testicle), and some received scrotal implants of bits of infected testicular tissue. In some animals the primary focus was removed by excision. All of the animals developed outspoken primary reactions and 21, or 95.5 per cent, showed metastatic lesions elsewhere.

The animals were divided into two groups. The first group (Group A) comprising ten animals, received six intravenous injections of 10 mg. per kilo of arsphenamine at weekly intervals. The second or control group (Group B) comprising twelve animals, received no treatment. Treatment was begun 127 days after inoculation, at a time when the acute phase of the infection had ended and the animals showed no manifestations of activity. Such lesions as were present were in the act of regressing.

A period of from 32 to 38 days was permitted to elapse following the last injection of arsphenamine, at the expiration of which both popliteal nodes were excised under ether anesthesia, ground up in sterile salt solution, and the resulting emulsion injected into the right testicle of each of two normal rabbits. Care was exercised to see that all of the emulsion was inoculated. These animals were observed over a period of 90 days before being discarded as negative. Any suspicious lesion developing in the inoculated testicle was aspirated and the material obtained was examined for treponemata with the dark-field illuminator.

Reinoculation of both treated and untreated groups was carried out 2 to 8 days after removal of the popliteal nodes and 40 days after treatment ceased, or 209 days after the original inoculation. For the purposes of reinoculation testicular emulsion containing actively motile treponemata of the same strain was used. The material was injected intradermally at the base of the right ear. An attempt was made to inject 0.1 cc. in each instance, but the results were not uniform, for owing to the delicacy of the skin in this region, the task proved to be a very difficult one. Almost invariably some of the material made its way into the subcutaneous tissue or leaked back along the needle tract. Fearing lest the experiment might be vitiated through failure to introduce a sufficient inoculum, a second attempt at reinoculation was carried out 9 days later. For this purpose the skin of the left ear was first shaved (without soap and water), the epidermis scarified with a scalpel until an area approximately 1 cm. square had been exposed, and a drop of testicular emulsion containing actively motile treponemata was gently

TABLE I.

Comparison of Results of Lymph Node Transfer in Arsphenamine Treated and Untreated Syphilitic Rabbits.

Group.	No. of rabbits.	No. of positive node transfers.	Percentage of positive node transfers.
Treated	10	0	0
Untreated	12	10	83.3

Treatment was begun 127 days after inoculation.

Transfers were carried out 194 to 195 days after inoculation and 32 to 38 days after treatment was completed.

rubbed in with the blade of the scalpel. It may be stated at this point that the subsequent course of events in the control animals proved that this precaution was unnecessary.

For the control of the activity of the virus used for reinoculation five normal rabbits were used. One of these died of an infection of the respiratory tract a few days after inoculation, but the remaining four survived long enough for the purposes of the experiment. Lymph node transfers were made from three of these virus control animals in order to be certain the infection had taken place. All three groups of animals, namely, treated, untreated, and virus controls were examined at regular and frequent intervals throughout a period of 90 days following the first reinoculation.

RESULTS.

The effect of treatment on the transmissibility of the infection by lymph node transfer is shown in Table I.

Table I shows that not one of the animals treated with six doses of arsphenamine each of 10 mg. per kilo harbored a sufficient number of treponemata in their popliteal lymph nodes to transmit the infection to normal rabbits, while of the twelve untreated controls ten, or 83.3 per cent, contained viable and virulent treponemata 194 to 195 days after inoculation. As judged by lymph node transfer all of the treated animals had been sterilized. In this connection it should be noted that when the lymph nodes were excised a striking difference in size was noted in the nodes of the animals of the two groups. Those in the untreated series were considerably and uniformly larger than those in the treated series.

TABLE II.

Results of Reinoculation of Treated and Untreated Syphilitic Rabbits.

Group.	No. of rabbits.	Positive.*		Doubtful.		Negative.	
		No.	Percent.	No.	Percent.	No.	Percent.
Treated	10†	0	0	0	0	10	100
Untreated	9‡	0	0	0	0	9	100
Virus controls	4	2	50	1	25	1	25

* By positive is meant production of a clinical lesion with demonstration of treponemata.

† One animal observed 54 days died from pneumonia.

‡ " " " 24 " " " "

The results of reinoculation are shown in Table II. It should be stated here that our criteria for successful reinoculation were the production, after suitable incubation period, of a clinically recognizable lesion and the demonstration of treponemata in the lesion. In some of the animals transitory inflammatory reactions occurring within a few days after inoculation were observed. These, however, were not regarded as evidence of a successful reinoculation since they might be explained as an allergic reaction, or as a simple foreign body reaction. In the table are presented the reinoculation results obtained at the site of the first reinoculation attempt (intradermal inoculation of the right ear). The results obtained at the site of the second reinoculation (scarification of left ear) must be excluded, since the behavior of the virus controls toward this mode of reinoculation

was such as to lead us to believe that the method cannot be relied upon. (Not one of the controls developed a characteristic lesion at the scarified inoculated area.)

In Table II only nine animals in the untreated series are reported upon. This is due to the fact that of the twelve animals originally in this group and reported upon in Table I, one died during the operative removal of the lymph nodes, one died of caseous pneumonia on the 16th day following reinoculation, too soon to judge of the result of this procedure, and the third developed paraplegia on the day following operation and had to be sacrificed. It may or may not be significant that the first two of these animals were the only ones in the group of twelve untreated rabbits whose popliteal nodes were not capable of transmitting the infection to normal animals.

Table II shows that in neither the treated nor the untreated group of rabbits was it possible to produce a successful reinoculation that would fulfill the requirements outlined above. It is necessary, at this point, to describe rather fully what took place in the control animals.

Of the four virus controls two, or 50 per cent, developed indurated papules at the site of inoculation after an incubation period of 16 days in each instance and actively motile treponemata could be demonstrated in the lesion in each animal. In one of these animals, No. 1, the lesion on the right ear attained a maximum diameter of 3 mm., underwent superficial necrosis, and finally healed spontaneously by the 40th day after inoculation. The animal died from an infection of the respiratory tract 66 days after inoculation. In the other, No. 2, the lesion attained a maximum size of 5 mm., was indurated and brownish red in color, showed superficial ulceration, and healed spontaneously about the 80th day after inoculation. The popliteal lymph nodes were removed from this animal 98 days after inoculation, emulsified in salt solution and injected into the testicles of two normal rabbits with positive results, thus establishing that infection had taken place in this animal.

A third animal, No. 3, showed a definite indurated area in the skin at the site of inoculation after an incubation period of 16 days. This lesion never exceeded 2 mm. in diameter and treponemata could not be demonstrated in the serum obtained from it. It healed spontaneously by the 54th day after inoculation. In the table this animal is entered as doubtful, but lymph node transfer carried out on the 98th day yielded positive results, demonstrating that infection had taken place and indicating that the lesion was syphilitic in nature.

In the fourth animal, No. 4, no lesion developed at the site of inoculation in the right ear at any time during the period of observation (99 days). However, lymph node transplantation from this animal to normal animals was also success-

ful, indicating that this animal likewise had been infected in spite of the fact that no lesion developed at the site of inoculation. This phenomenon has been observed before in rabbits (11, 12) and Neisser has observed the same thing happen in monkeys.

The results in the four control animals leave no doubt as to the infectivity of the virus employed for reinoculation. Yet neither the untreated nor the treated syphilitic rabbits showed any evidence that infection had been accomplished as judged by the production of a characteristic lesion at the site of the reinoculation.

DISCUSSION.

These experiments show clearly that the administration of arsphenamine intravenously to rabbits in doses of 10 mg. per kilo to the extent of six doses spaced at intervals of 1 week is sufficient to eliminate an existing syphilitic infection, as judged by lymph node transfer performed 32 to 38 days after cessation of treatment, even if the infection has been present for a period of 127 days when treatment is begun. Our results are in complete agreement with those of Nichols and Walker, who obtained similar results with even fewer administrations of the drug, lymph node transfer carried out as long as 61 days after treatment remaining negative in the hands of these investigators. It has been clearly established, therefore, that arsphenamine has the property of abolishing lymph node infection with *Treponema pallidum* in the rabbit according to the most rigid tests now available.

Whether or not such animals continue to harbor virulent treponemata in other less accessible portions of the body, or at later periods in lymph nodes, is still debatable, but at least it can be stated that a long standing infection of lymph nodes can be eradicated by proper attack, as far as can be judged at present.

The interpretation of the results obtained with the attempts at reinoculation is more difficult. Granting that the virus controls were sufficient to indicate the potency of the virus, and the production of local lesions together with dissemination of the virus would seem to prove such potency, then it may be said that the treated and untreated syphilitic animals behaved in a manner altogether different from that of the normal controls. They showed no clinical evidence

of infection having been established at the reinoculation site. Judged by the absence of any lesion at the portal of entry the two groups of animals, both treated and untreated alike, were entirely refractory to a second infection.

The question naturally arises is this absence of any local lesion at the site of inoculation to be regarded as evidence that infection has not taken place? In view of the now clearly established fact (12) that rabbits may be infected with *Treponema pallidum* by intradermal inoculation without the production of any visible reaction at the site of inoculation, it seems justifiable to raise this query. However, if any large proportion of animals in either group were susceptible of infection by intradermal inoculation it is highly unlikely, indeed, that such susceptibles would all have been infected without the production of any local lesion whatsoever at the site of inoculation. It seems much more likely that at least one or two would have shown something at the site of inoculation comparable to that seen in the virus control group. On the whole, then, we are inclined to the view that the animals in both the treated and untreated groups were in reality not reinfected and that they did not exhibit a local lesion at the site of inoculation because they were in truth refractory. In saying this we are fully aware that the ultimate proof that these animals were not reinfected has not been brought. Experiments designed to test this point are in progress at the present time.

If they were not reinfected, as seems likely to us, it is interesting to speculate upon the bearing of that fact upon the question of whether or not these animals are to be regarded as harboring active syphilitic virus. In the past it has been assumed that failure to reinfect an animal previously inoculated with *Treponema pallidum* meant the presence of active syphilis in such an animal. The experimental basis for this view was laid by the work of Neisser and his collaborators, and that view has been generally accepted as true and has been applied to the experimental evaluation of antisyphilitic agents. However, the behavior of our treated animals toward reinoculation raises the question as to the validity of this method as one adapted for determination of a biological cure. For the group of treated animals had been shown to have been previously sterilized so far as their lymph nodes were concerned, yet they gave

evidence of being refractory to a second inoculation. Although it is impossible to say whether or not they had been rid of *all* their treponemata by the arsphenamine therapy, nevertheless, the probabilities are strong that such was the case; and yet, at a time when they were known to be free of popliteal lymph node infection, they were still refractory to a second inoculation. Is this refractory state to be attributed to the presence of treponemata in some other and altogether inaccessible portion of the body, in other words, to a still existing infection? May it not be explainable upon the basis that the treated animals had, through the existence of the infection over a fairly long period of time, acquired such a state of resistance that even after eradication of the treponemata by suitable therapy the animals still maintained that state of resistance as expressed by failure to show any clinical phenomena on attempts at reinoculation?

In other words, is failure to reinoculate animals already infected and treated, to be interpreted as failure to "cure?" It seems to us that in the light of our experiments such a failure to reinoculate could equally well be interpreted as evidence that the animal was immune and not necessarily still infected, although it might so be. Neisser admitted the possibility of such a condition but stated that he had not encountered any evidence that it actually occurred.

Such a view fits in with the facts thus far observed, and is entirely in harmony with Kolle's experiments. He obtained uniformly successful reinoculations if treatment was carried out before the 45th day of infection. The results were less constant if the treatment was begun between the 45th and 90th day of infection, while if it was begun after the 90th day no successful reinoculations were obtained. Kolle interpreted these results as being evidence for the view that the treatment had failed to cure the infection. It seems to us quite as likely that these results may be explained on the basis of the animals having had the infection for a sufficiently long period of time to have acquired a high degree of refractoriness toward a second inoculation. In other words, they might well have been cured and an interval of 90 days may have been sufficient for the establishment of the refractory state. It seems to us that this interpretation of the experimental facts recorded above has as much to recommend it as the other, namely, that the refractory state is due to a still existing infection.

In brief, we are inclined to the view that the demonstration of the presence or absence of lymph node infection affords a better criterion for the evaluation of an antisyphilitic agent than does the method of reinoculation, as practised in our experiments. We wish, also, to point out that the results of our experiments suggest that failure to reinoculate treated syphilitic rabbits does not necessarily indicate the presence of infection in the latter, but may, on the other hand, indicate the existence of an acquired immunity.

A fundamental objection to the reinoculation method as a criterion of therapeusis is the difficulty of being certain that infection has not taken place when no local lesion is produced at the site of inoculation. In such instances there is always the possibility that infection has occurred without there being apparent at the portal of entry any clinical phenomena which would indicate that such had occurred. As Brown and Pearce (13) have aptly expressed it, in judging attempts at reinoculation one must be able to see beyond the reaction at the site of inoculation. Another and quite as serious objection to the reinoculation method is the difficulty in controlling the virulence of the virus. This difficulty is inherent in all experimental work in syphilis at the present stage of its development and renders it well nigh impossible to compare one set of experiments in which one batch of virus is used with another set performed with a different batch even of the same strain. There is, also, the strong probability that the manner in which the second inoculation is carried out (site or mode) may play an important rôle in the initiation and development of a second infection. This phase of the question has received some attention already, but it has not been definitely settled thus far.

CONCLUSIONS.

1. The intravenous administration of six doses of arsphenamine to syphilitic rabbits in amounts of 10 mg. per kilo, 127 days after inoculation is sufficient to render the popliteal nodes of such animals incapable of transmitting the infection to normal animals.

2. Syphilitic rabbits that have been treated in this manner and whose popliteal nodes 32 to 38 days after treatment have been shown to be incapable of transmitting the infection to normal animals are, as far as can be judged by the absence of a local lesion, refractory to

a second inoculation of active virus of the same strain, if the inoculation be made intradermally at the base of the ear and carried out 209 days after the first inoculation.

3. Untreated syphilitic rabbits are also refractory toward a second inoculation made in a similar manner and at a similar interval following the first inoculation.

4. Evidence is offered in favor of the view that the most satisfactory method of evaluation of antisyphilitic agents in experimental syphilis of the rabbit is that of lymph node transfer.

5. Evidence is offered in support of the view that the state of refractoriness toward a second inoculation exhibited by syphilitic rabbits may be explainable upon the basis of either an acquired immunity or persistence of a focus of living treponemata in the body.

BIBLIOGRAPHY.

1. Ehrlich, P., and Hata, S., Die experimentelle Chemotherapie der Spirillosen, Berlin, 1910.
2. Kuznitsky, E., in Neisser, A., Beiträge zur Pathologie und Therapie der Syphilis, Berlin, 1911, 295.
3. Neisser, A., Beiträge zur Pathologie und Therapie der Syphilis, Berlin, 1911, 182.
4. Uhlenhuth, P., and Mulzer, P., *Beitr. exp. Path. u. Therap. Syph.*, Berlin, 1913, 151.
5. Kolle, W., *Deutsch. med. Woch.*, 1922, xlviii, 1301.
6. Frei, W., *Arch. Dermatol. u. Syph.*, 1923, cxliv, 365.
7. Pearce, L., and Brown, W. H., *J. exp. Med.*, 1922, xxxv, 39.
8. Hill, J. H., and Young, H. H., *J. Am. Med. Assn.*, 1923, lxxx, 1365.
9. Nichols, H. J., and Walker, J. E., *J. Exp. Med.*, 1923, xxxvii, 525.
10. Voegtlin, C., Armstrong, C., and Dyer, H. A., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 1815.
11. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 200.
12. Chesney, A. M., *J. Exp. Med.*, 1923, xxxviii, 627.
13. Brown, W. H., and Pearce, L., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 255.

THE CORRELATION OF CERTAIN PHENOMENA OCCURRING DURING THE GROWTH OF PNEUMOCOCCUS.

By HUGH J. MORGAN, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

(Received for publication, September 25, 1923.)

In the course of investigations undertaken in this laboratory it became desirable to correlate in one experiment certain growth phenomena of pneumococcus in plain broth and to ascertain their time relationships as expressed in terms of the growth curve. It was shown by Chesney (1) that the growth curve of pneumococcus in plain broth varies but little. Avery and Cullen (2) observed that pH changes occurred in the substrate of such cultures. Dochez and Avery (3) noted the presence of a substance in the substrate of pneumococcus cultures which reacts specifically with the type anti-pneumococcus serum. It is known that under certain conditions peroxide occurs in pneumococcus cultures (4, 5) and that bacteria-free filtrates containing this compound promptly change solutions of catalase-free crystalline hemoglobin to methemoglobin.[1] Moreover, it has been shown (6) that when the filtrate of a broth culture of pneumococcus is reinoculated with pneumococcus visible growth does not occur, and that this is due to at least three factors: the first is the unfavorable pH of the filtrate; the second is the lack of sufficient nutritive substances; the third is the presence of a peroxide in the filtrate. It has been demonstrated that a hardier organism, as *Staphylococcus aureus*, will grow in pneumococcus filtrates despite the action of the first two factors; whereas, growth does not occur in filtrates containing peroxide.

An attempt was made to investigate the time relations of the above phenomena.

[1] Unpublished observations. The relation of catalase to this phenomenon and the destruction of oxyhemoglobin by pneumococcus under other conditions will be discussed in a subsequent communication.

EXPERIMENTAL.

Method.

Into a large Erlenmeyer flask were placed 450 cc. of plain broth. The flask was of such size that the broth in it reached a depth of only 2 cm., thus insuring a large surface area-volume ratio (5). The medium was incubated for 24 hours to insure sterility and then seeded with 0.5 cc. of a 6 hour broth culture of Pneumococcus Type II. It was replaced in the incubator and at intervals of a few hours 30 cc. fractions of the culture were removed for examination.

The procedure for the examination of each fraction was as follows: A measured amount was plated in glucose agar in order to determine the colony count. The remainder was passed through a Berkefeld filter. A colorimetric pH determination was made upon a sample of the filtrate thus obtained. 0.5 cc. of the filtrate was added to an equal quantity of Type II antipneumococcus serum to test for the presence of the specifically reacting (soluble) substance. The presence or absence of peroxide was determined as follows: 2 drops of a freshly prepared saturated solution of benzidine in glacial acetic acid were added to 0.5 cc. of the filtrate. This was done in an agglutination tube containing a piece of raw potato about 2 cm. square.[2] With a 2 per cent solution of hemoglobin made from crystalline hemoglobin containing no catalase,[3] a test was then performed for the transformation by the filtrate of oxyhemoglobin to methemoglobin. Equal quantities of the oxyhemoglobin solution and filtrate were used and the macroscopic readings were verified by spectroscopic examination. All readings were made after the tubes had remained in the water bath at 37°C. for 30 minutes. With the remainder of the filtrate cultivation experiments were performed. The fractions showing a change in pH, due to the growth activity of the pneumococcus, were readjusted to 7.7. Into 5 cc. quantities of this readjusted pneumococcus culture filtrate Pneumococcus Type II and *Staphylococcus aureus* were seeded. The inoculum in the case of the pneumococcus consisted of 0.05 cc. of a 6 hour broth culture and in the case of the staphylococcus of one loop of a 6 hour broth culture.[4]

---

[2] The presence of peroxide is promptly indicated in this test by the appearance of a blue color about the potato; when the reaction is intense the surrounding fluid, as well as the potato, shows the color change.

[3] We are indebted to Dr. Michael Heidelberger for the crystalline hemoglobin prepared from horse blood.

[4] These cultures were kept in the ice box throughout the experiment and at its completion their viability was controlled by transfers into plain broth, with the standard inocula. There was abundant growth at the end of 6 hours in each instance.

Growth Activity, as Determined by Colony Count, of the Broth Culture of Pneumococcus Type II.

The rate of growth of the pneumococcus culture was determined by a numerical estimation of the number of viable organisms in the culture fluid at different periods of growth.

The number of organisms present in 1 cc. of the culture was determined by the colony count method, with 1 per cent dextrose agar. Dilutions of the culture were carried out by 10's and were so made that 0.5 cc. of the fluid of a given dilution contained a known amount of the original culture. Separate pipettes were used in making the dilutions. Two or more dilutions of the culture were plated in each

TABLE I.

Correlation of Phenomena Occurring during Growth of Pneumococcus.

Time after seeding.	No. of colonies per cc. of culture.	pH of subtrate.	Precipitin reaction with antipneumococcus serum (Type II).	Presence of peroxide.†	Formation of methemoglobin from oxyhemoglobin.‡
hrs.					
0	3,960	7.7	−*	−	−
4	273,000	7.7	−	−	−
7	8,600,000	7.7	+	−	−
10	50,500,000	7.4	++	+	+
13	450,000,000	7.3	+++	++	++
16	179,000,000	7.3	+++	+++	+++
19	138,000,000	7.2	+++	+++	+++
27	50,000	7.2	+++	+++	+++

* − indicates no reaction; +, visible precipitate; ++, flocculation; +++, heavy flocculation.

† Determined by the benzidine reaction. − indicates no reaction; +, blue color about the piece of potato; ++, blue color of potato and to a less extent of surrounding fluid; +++, intense blue color of potato and surrounding fluid.

‡ Prepared from crystalline hemoglobin containing no catalase.

examination and, making the necessary calculation for the different dilutions, the average of them was used as the colony count per cubic centimeter of culture for that hour. The count was made after the dextrose agar plates containing the different dilutions of the culture had remained in the incubator at least 48 hours.

The usual period of lag was avoided by the use of a relatively large inoculum from an actively growing culture. It is seen from Table I that the number of colonies per cubic centimeter increased

steadily during the first 13 hours of incubation. Examinations made after this time revealed a rapidly decreasing colony count until the 27th hour, at which time only 50,000 colonies grew from 1 cc. of the culture.

Reaction Changes in the Broth Culture Filtrates of Pneumococcus Type II.

The changes in pH which occurred in the substrate during growth of the broth culture of pneumococcus were noted.

The pH readings were made by the colorimetric method and were determined on the filtrates of fractions removed from the parent culture at stated intervals (Table I).

It is evident from Table I that the pH of the broth remained practically unchanged (pH 7.7) during the first 7 hours of incubation. Between the 7th and 10th hours the medium became less alkaline, the reading made at the end of the 10th hour being 7.4. After the 10th hour the pH slowly decreased until, at the end of the experiment (27 hours of incubation), the reading was pH 7.2.

The Occurrence of Specific Soluble Substance in the Broth Culture Filtrates of Pneumococcus Type II.

The time of appearance of the specifically reacting substance was determined by examination of the bacteria-free filtrates of the culture at different phases of its growth.

At the intervals stated in Table I, 0.5 cc. of the culture filtrate was added to 0.5 cc. of Antipneumococcus Serum Type II. The tubes containing the mixtures of immune serum and culture fluid were incubated in the water bath at 37°C. for 1 hour, and the presence or absence of specific precipitation was noted (Table I).

The occurrence of the soluble substance was first noted in the filtrate obtained from the culture after 7 hours of growth. Although no quantitative titration of the precipitinogen was made the maximum reaction was reached by the 13th hour and persisted in all subsequent tests.

The Occurrence of Peroxide in the Broth Culture Filtrates of Pneumococcus Type II.

The time of appearance of peroxide in the culture of pneumococcus was determined by examining filtrates of the culture at different intervals of growth.

0.5 cc. of the filtrate of the fraction of culture to be examined was placed in an agglutination tube. A small piece of raw potato was then dropped into the tube and immediately 0.1 cc. of the benzidine reagent was added. The appearance of a blue color indicated the presence of peroxide in the filtrate. Immediate readings of the tests were made since the color reaction tends to disappear on standing (Table I).

The peroxide test was negative in the fractions removed at the 4th and 7th hours of incubation of the culture. However, the filtrate of the culture after 10 hours growth gave a positive reaction. This reaction increased in intensity, reaching its maximum at the 16th hour, and it remained strongly positive throughout the remainder of the experiment.

Methemoglobin Formation by the Broth Culture Filtrates of Pneumococcus Type II.

Tests were made at intervals during growth of the pneumococcus culture to determine whether or not the filtrate possessed the property of converting solutions of crystalline hemoglobin to methemoglobin.

The hemoglobin consisted of a 2 per cent solution of catalase-free crystalline hemoglobin in phosphate solution pH 7.8. 0.5 cc. of this hemoglobin solution and 0.5 cc. of the filtrate to be tested were pipetted into an agglutination tube and placed in a water bath at 37° for 30 minutes. Control experiments consisting of equal quantities of plain broth and hemoglobin solution were made with each filtrate. The macroscopic readings were verified in each instance by spectroscopic examination.

As is shown in Table I, solutions of crystalline hemoglobin containing no catalase were converted into methemoglobin by the bacteria-free filtrates of the pneumococcus culture. This phenomenon did not occur with filtrates of fractions of the culture obtained before or at the end of the 7th hour of incubation but

appeared first in the fraction which was removed after 10 hours of incubation. By the 16th hour of incubation the methemoglobin reaction had apparently reached the maximum, and so persisted throughout the remainder of the experiment.

Growth of Pneumococcus and Staphylococcus in Filtrates of a Broth Culture of Pneumococcus Type II.

Cultivation experiments, with the homologous organism, Pneumococcus Type II, and *Staphylococcus aureus*, were made with the filtrates of the fractions of the pneumococcus culture. These fractions were removed at different periods during the growth of the pneumococcus culture.

TABLE II.

Growth of Pneumococcus and Staphylococcus aureus in Filtrates of a Broth Culture of Pneumococcus Type II.

Filtrate of pneumococcus broth culture after incubation.	Growth of Staphylococcus R in pneumococcus filtrate (pH 7.8).					Growth of Pneumococcus Type II in pneumococcus filtrate (pH 7.8).				
	12 hrs.	24 hrs.	48 hrs.	96 hrs.	168 hrs.	12 hrs.	24 hrs.	48 hrs.	96 hrs.	168 hrs.
hrs.										
0	+					+				
4	+					+				
7	+					+				
10	0	0	0	+		0	0	0	0	0
13					0*					0
16					0*					0
19					0*					0
27					0*					0

* A blood agar plate inoculated with one loop from this tube remained sterile.

The pH of the filtrate of each fraction of the culture was determined and, when necessary, readjusted to 7.7. One loop of a 6 hour broth culture of *Staphylococcus aureus* and 0.05 cc. of a 6 hour broth culture of Pneumococcus Type II, respectively, were seeded into 5 cc. portions of the filtrates. The presence or absence of growth was determined macroscopically (Table II).

It is apparent from Table II that growth occurred when the homologous strain of pneumococcus was seeded into the filtrates of fractions of the pneumococcus culture removed before or at the end of 7 hours incubation. Filtrates of fractions removed from the culture

after it had incubated for 10 hours or longer failed to support growth, as determined by macroscopic examination.

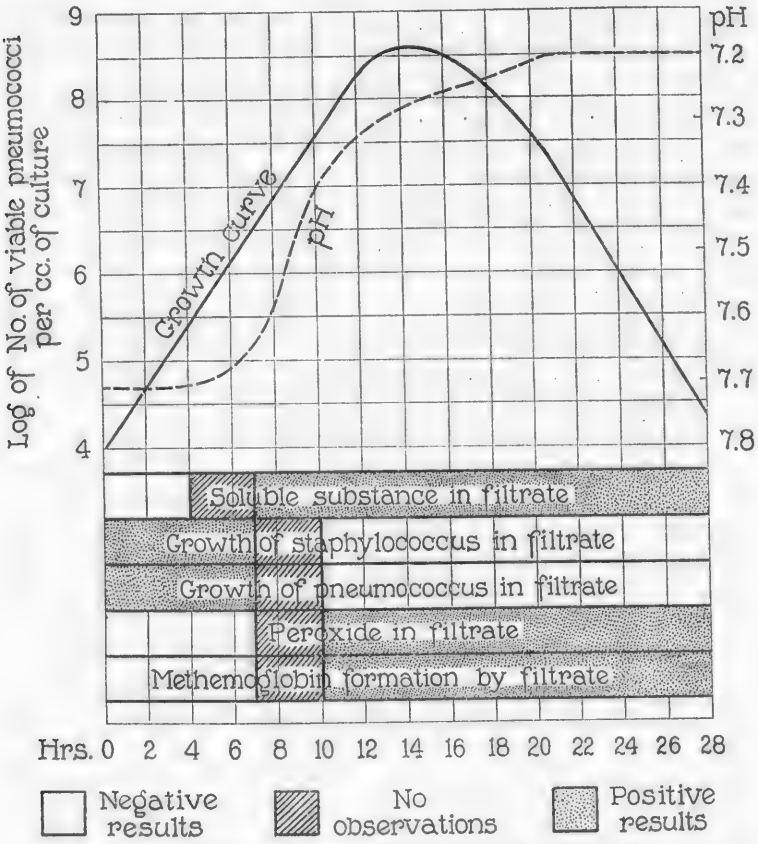
Staphylococcus grew rapidly and abundantly in the filtrates of those fractions of the pneumococcus culture which were removed before, and at the end of 7 hours of incubation. Only after 4 days did growth of staphylococcus occur in the filtrate of the fraction which was removed after 10 hours of incubation. Staphylococcus failed to grow in filtrates of the pneumococcus culture when the latter had been incubated 13 hours or longer. Moreover, the staphylococci introduced into these filtrates by the inoculum were no longer viable after 7 days of exposure to this environment.

DISCUSSION.

In a series of observations upon an individual culture of pneumococcus it has been possible to demonstrate within a single experiment many of the physiologic activities of the growing cell. These observations include a numerical estimation of the number of viable organisms at different periods of growth and the correlation of growth activity with the production of acidity, the elaboration of a soluble specific substance, the formation of peroxide and, under certain conditions, the conversion of oxyhemoglobin to methemoglobin. The results of the various examinations made during the course of the experiment are represented graphically in Text-fig. 1. By a study of this chart one is enabled to ascertain the time relationship of some of the phenomena which occur during the growth of the pneumococcus, under the conditions described. The significance of the occurrence of these growth products in culture fluids and their relation to accompanying phenomena such as growth inhibition has been pointed out in previous papers. While in these earlier studies the experimental facts concerning these substances have been given in detail, it seemed desirable to comprise within a single experiment observations which would summarize the relationships existing between these several growth phenomena of pneumococcus.

From the graphic presentation of results given in Text-fig. 1, it is evident that under appropriate conditions, the rate of growth of pneumococcus follows the characteristic curve previously described by Chesney. It is further evident that during the time of maximum

rate of growth, that is during the period of logarithmic increase when cell multiplication is at its maximum, there is a critical phase in which these various products of cell metabolism begin to accumulate



TEXT-FIG. 1. Correlation of phenomena occurring during growth of pneumococcus; time relations are expressed with reference to the growth curve.

in the culture fluid. Although the exact hours given in the present experiment are relative and largely dependent upon the particular cultural conditions, they serve, nevertheless, to indicate the ap-

proximate time of appearance and duration of the various phenomena studied. While under other circumstances the exact time relationships may be modified, the appearance of these substances expressed in terms of the growth phases of the culture should remain constant. The experimental data presented justify the conclusion that these changes in the bacterial substrate appear relatively early in the logarithmic phase of growth. In the present experiment in which bacterial lag was purposely avoided, this critical time in the age of the culture appears to lie between the 7th and 13th hours of incubation. At the beginning of this period there is a progressive increase in the number of organisms, and within this period the reaction of the medium becomes progressively more acid with a change in pH from 7.7 to 7.3, which is within 0.1 pH of the maximum acidity finally reached. Concomitantly with this active cell multiplication and reaction change, there appears in the culture fluid at about the 7th hour the so called soluble substance which reacts specifically with antipneumococcus serum of the homologous type. This specifically reacting substance increases with the increasing growth and persists during the final stages of dissolution of the bacterial cells. As previously pointed out (3), the early appearance of this substance in soluble form indicates that it is the product of cell activity rather than of cell death.

During the critical phase of growth, in addition to the changes already noted there appears in solution in the culture fluid another substance, which has the properties of hydrogen peroxide (4). This peroxide is demonstrable by the 10th hour of growth and gradually increases in concentration until the maximum is reached at about the 16th hour. With its appearance the bacteria-free filtrates of the culture acquire for the first time the property of transforming catalase-free crystalline hemoglobin to methemoglobin, and of inhibiting the growth of other organisms such as *Staphylococcus aureus*. The relationship between peroxide and methemoglobin formation under the conditions of this experiment seems to be one of cause and effect, since hydrogen peroxide even in minimal concentration is capable of converting oxyhemoglobin into methemoglobin providing the solution of blood pigment is prepared from crystals of hemoglobin in which catalase has been destroyed. As

pointed out earlier in the present paper, simultaneously with the occurrence of peroxide in the medium the culture filtrates acquire the property of inhibiting growth. That the inhibition is dependent, in the case of *Staphylococcus aureus* at least, upon the presence of peroxide, is indicated by the fact that the same culture fluids, after the peroxide has been destroyed, suffice to support growth of this organism (6). By contrast the pneumococcus itself, of the same or of different type, is unable to grow in the filtrate of a culture in which it previously thrived. This autoinhibition is dependent upon factors other than the presence of peroxide, since in the absence of this substance growth cannot be initiated in a medium previously metabolized by pneumococcus. Acidity alone is not accountable for failure of growth under these conditions, since on reinoculation of culture filtrates, the reaction of which has been readjusted to the optimal pH, growth of pneumococcus still does not occur. Obviously then the growth is restrained by other factors besides acidity and peroxide, both of which, however, may under certain circumstances limit it. This third factor involved in the mechanism of growth inhibition, as has been previously pointed out, seems to be the absence of fermentable substance in the medium, a condition due to specific exhaustion by previous growth. For it has been shown that the addition of small traces of sugar to plain broth previously metabolized by pneumococcus suffices to render the medium again suitable for growth of this organism (2).

SUMMARY.

The growth of a culture of pneumococcus was followed together with the time relations of some of the phenomena incident thereto. There were, roughly speaking, three periods of growth: first, the period of maximum rate, or logarithmic period; second, a brief stationary period; and third, the period of decline, during which actual death of individual organisms in the culture occurred. It has been pointed out that the usual preliminary latent period was avoided by the use of relatively large inoculum, obtained from a culture which was itself in the logarithmic period of growth.

During the height of growth activity of the culture certain changes in the substrate became demonstrable. Between the 7th and 10th

hours the reaction of the medium was changed by the production of acid and the soluble specific substance was detected. At a somewhat later time in the logarithmic period of the culture peroxide was demonstrable and, simultaneously with the appearance of this substance, the substrate acquired the ability to change catalase-free crystalline hemoglobin to methemoglobin. Moreover, changes then occurred in the cultural fluid, such that, after the removal of the parent organisms by filtration, it no longer supported growth when seeded with either the pneumococcus or staphylococcus. These changes occurred early in the logarithmic period of growth of the culture.

BIBLIOGRAPHY.

1. Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.
2. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxx, 359.
3. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1917, xxvi, 477.
4. McLeod, J. W., and Gordon, J., *Biochem. J.*, 1922, xvi, 499.
5. Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1924, xxxix, 275.
6. Morgan, H. J., and Avery, O. T., *J. Exp. Med.*, 1924, xxxix, 335.

THE INTERACTION OF TWO FRAGMENTS OF PULSATING HEART TISSUE.

By ALBERT FISCHER, M.D.

(From the University Institute for General Pathology, Copenhagen, Denmark.)

PLATE 28.

(Received for publication, September 27, 1923.)

It has seemed possible that a synchronous contraction could be obtained *in vitro* of two different, separated fragments of heart, if anastomosis between the autonomic contractile elements could be established. In the present paper experiments are reported on the theme.

Technique.

First the optimal conditions were determined under which fragments of embryonic heart tissue from chicken continue to pulsate *in vitro*. The best results were obtained when the plasma was diluted with Ringer or Tyrode solution to about 20 per cent. One volume of a 20 per cent plasma in Ringer solution and one volume of fresh embryonic tissue juice were the composition of the culture medium.

The fragments of heart were obtained from chicken embryos approximately 8 to 10 days old. In some of the experiments the fragments came from different embryos, and in some they were derived from the same heart, while in yet others two fragments derived from the same heart had their cut edges brought into close approximation. This was accomplished in the following way. A rather large fragment of heart was transferred to the culture medium and while the plasma and embryonic juice were still in a liquid state, the fragment was divided with a clean cut and the two pieces kept apposed by means of a preparation needle and the point of a cataract knife until coagulation of the plasma had taken place.

Usually three fragments were placed in one culture, two adjacent to each other and one separately as a control.

A couple of hours after the preparation of the cultures, the rate of contractions of the various fragments was recorded. The interval of time between two contractions was checked for the control (the isolated fragment) and for each of the two adjacent fragments, by means of a stop-clock. The observations were repeated on the 3 to 4 days following, after which the heart fragments ceased to pulsate.

The distances between the two adjacent fragments in the culture were purposely varied from direct contact to about the width of one of the fragments, *i.e.* about 1 mm.

Results.

When several fragments of heart tissue were embedded in the same culture medium, no two of the pieces pulsated at the same rate. The intermission between successive contractions was the same for any given piece but not like that for others.

In experiments in which the distance between two adjacent fragments was about 1 mm., it was noticed that the intermission between two contractions remained constant over a long period of time. This rhythmic contraction for the individual fragments lasted about 20 hours by which time the outgrowth of new cells, mainly fibroblasts, had bridged the zone between the two pieces (Fig. 1). From now on the pulsation became irregular. Long intermissions were followed by periods of quick contraction or of fibrillar contractions (Table I). Meanwhile the isolated fragment in the same culture which served as a control had continued to pulsate with a constant time interval. The culture was now unsealed and by means of a clean cut with a sharp cataract knife the two adjacent bits were separated in the zone of new growth. The culture was resealed and again observed under the microscope. The procedure of separation lasted only about 1 minute. From now on, the contractions of the two pieces were rhythmic, *i.e.* the intermission between the contractions was constant for each of the two pieces but not synchronous (Table I).

The interpretation of this phenomenon is difficult. It may perhaps be explained by a mechanical interference with contraction after the fragments have grown together.

When the two fragments of heart tissue had been placed very close to each other in the culture they pulsated as one, that is to

TABLE I.

Culture 2013.

Time between two contractions of control.	Time between two contractions of one of two adjacent fragments.	Time between two contractions of the other.
Before the two became connected.		
	sec.	*sec.*
	2.0	1.6
	2.0	1.6
	2.0	1.6
	2.0	1.4
	2.0	1.6
After the connection.		
sec.		
3.0 3.0	1.5 2.0	1.2 1.0
3.0 3.0	2.0 2.5	20.0 0.8
3.0 3.0	2.0 2.0	1.0 0.6
3.0 3.0	2.0 1.8	0.8 0.8
3.4 3.0	2.1 1.5	1.0 0.8
3.0 3.0	1.5 2.0	35.0 1.0
3.0 3.0	1.5 2.0	1.0 0.6
3.0 3.0	1.5 1.5	0.8 0.4
3.0 3.4	1.5 1.5	0.6 0.6
3.0 3.0	1.5 1.8	1.0 0.8
3.0 3.0	1.8 1.8	0.8 0.4
	1.5 1.5	18.0 0.4
	1.8	1.0 1.0
After separation.		
	5.0 5.0	8.0 8.0
	5.0 5.0	8.0 8.0
	5.0 5.0	8.0 8.0
	5.0 5.0	8.0 7.0
	5.0 5.0	7.0 7.0
	5.0 5.0	7.0 7.0
	5.0 5.0	7.0 7.0
	5.0 5.0	7.0 7.0
	5.0 5.0	7.0 7.0
	5.0 5.0	7.0 7.0
	5.0 5.0	8.0 7.0
	5.0 4.0	8.0 8.0

Greatest difference before separation, 1.0.....34.6
Average difference before separation, 0.2.....5.9

Greatest difference after separation, 1.0.....1.0
Average difference after separation, 0.0.....0.1

say synchronously, after about 48 to 72 hours incubation at 39°C. The space between them could scarcely be made out after 48 hours in instances in which the fragments pulsated absolutely synchro-

TABLE II.

Experiment 2040.

Time.	Time between two contractions of one of two adjacent fragments.	Time between two contractions of the other.
hrs.	*sec.*	*sec.*
0	1.6	2.4
0	1.6	2.4
0	1.6	2.0
0	1.6	2.0
0	1.8	2.0
0	1.4	2.0
0	1.4	2.0
0	1.4	2.0
24	1.2	3.4
24	1.2	3.6
24	1.4	3.8
24	1.8	4.0
24	3.0	2.4
24	2.2	2.2
24	4.6	2.0
24	4.2	1.6
	Interval between the synchronous contractions.	
48		
48	3.0	
48	3.2	
48	3.0	
48	3.4	
48	3.6	
48	4.2	
48	2.6	
48	3.0	
48	3.0	

nously. The data of such an experiment are given in Table II. The synchronism in this case was first obtained after 48 hours. The experiment also typifies the results with fragments of heart derived from two different chick embryos.

A rather high percentage of success in obtaining synchronous pulsation of fragments of heart resulted when two cut edges were brought directly in contact with each other and especially when those resulting from the same cut were apposed. For the latter purpose a piece of heart was cut in two in culture medium already prepared

TABLE III.

Experiment 2066.

Time.	Time between two contractions of one of two adjacent fragments.	Time between two contractions of the other.
hrs.	*sec.*	*sec.*
0	1.2	4.0
0	1.2	3.8
0	1.2	4.0
0	1.2	4.0
0	1.2	4.0
0	1.0	4.0
0	1.0	4.0
0	1.0	4.0
	Interval between the synchronous pulsations.	
24		
24		4.0
24		0.2
24		0.1
24		0.1
24		0.1
24		0.1
24		0.1
24		0.1
24		0.1
24		4.2
24		4.0
24		0.1
24		0.1
24		0.1
24		0.1

and the pieces kept in contact until coagulation took place. By this method a synchronous pulsation could be established within 24 hours (Table III).

A good many experiments were made to see if it was possible to obtain synchronous contractions between two fragments of heart,

one from a duck embryo and the other from a chicken embryo. Duck embryo tissue from the heart can be successfully cultivated *in vitro* in a heterologous culture medium composed of chicken plasma and chick embryo juice. The growth was just as extensive as that of the chicken tissue. Fragments of heart from duck embryo were able to pulsate vigorously through several passages in the chicken culture medium. It was possible, therefore, to determine whether tissues of the same organ derived from different species were able to unite and become a physiological unit. The result of these experiments was that no synchronous contractions could be obtained between fragments of heart, one of them from duck and the other from chicken embryo.

Serial sections were made of the cultures. A definite histologic difference could be observed in the zone between fragments that had pulsated synchronously and others that had not. In the line of contact of the latter, the contractile elements were separated by a layer of connective tissue (Fig. 1), whereas in the case of the fragments contracting together, the contractile elements from one had established a contact with those of the other (Fig. 2).

DISCUSSION AND SUMMARY.

Fragments of heart from the same species, when cultivated *in vitro*, are capable of uniting and pulsating synchronously. This happens not only in fragments derived from the same heart but with those from different individuals of the same species. It was not possible, on the other hand, to obtain, under the conditions of experiment, any physiological union of a heart fragment from a duck embryo with one from a chicken embryo, though both pulsated in the same medium and though the tissue cells of the duck were capable of growth and multiplication.

From the experiments it is evident that cell contact is essential for the development of physiological identity as just described. Since such a physiological identity cannot be obtained through the union of a fragment of a duck heart and a fragment of a chicken heart, one may suppose that anastomosis between the cells must play a very significant rôle in it.

CONCLUSIONS.

1. It is possible to establish a physiological union *in vitro* of two fragments of pulsating heart from the same species, such that a synchronous pulsation of them is obtained.

2. It has proved impossible to obtain synchronous pulsation in two fragments of heart belonging to different species (duck and chicken).

3. The facts indicate that cellular anastomosis between the contractile elements of the fragments originally separate are the cause for the physiological union of the two.

EXPLANATION OF PLATE 28.

FIG. 1. Experiment 2067. A section through the line of contact of two fragments of chick embryo heart which failed to pulsate synchronously *in vitro*. A layer of connective tissue can be seen separating the contractile elements. × 175.

FIG. 2. Experiment 2066. A section through the line of contact of two fragments of chick embryo heart which had pulsated perfectly synchronously *in vitro*. The contractile elements of the one are in direct contact with those of the other. × 175.

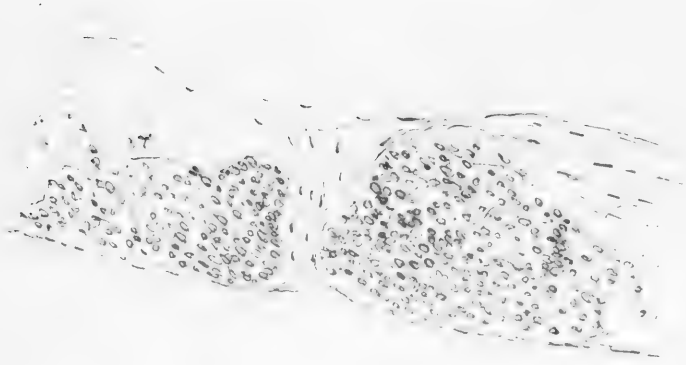


FIG. 1.

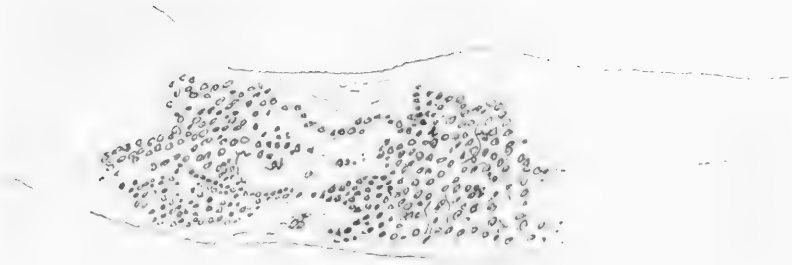


FIG. 2.

(Fischer: Interaction of pulsating heart tissue.)

UNIV. OF MICH.

THE DIFFERENTIATION AND KERATINIZATION OF EPITHELIUM IN VITRO.

By ALBERT FISCHER, M.D.

From the Laboratories of The Rockefeller Institute for Medical Research and the Institute for General Pathology of the University of Copenhagen, Copenhagen, Denmark.

PLATE 29.

(Received for publication, October 29, 1923.)

It has been stated by Champy[1] that epithelium from embryos, when cultivated *in vitro*, dedifferentiates within a few hours, whereas adult tissue persists for a long time in cultures without changing. He also stated that the dedifferentiation of epithelial cells is delayed, or does not take place at all, when fibroblasts or connective tissue cells are present. The same statement has been made quite recently by Drew.[2] He described a pure culture of epithelium which commenced suddenly to differentiate when connective tissue cells were added. By differentiation is meant here formation of rudimentary tubules. These are formed by normal epithelium and by that which is cancerous as well. Drew[2] describes, however, the growth of epithelium as occurring in "sheets of undifferentiated tissue." Champy means by dedifferentiation of the epithelial cells, a transformation into indifferent embryonic tissue, not unlike fibroblasts. It is therefore not quite correct of Drew to call the epithelium growing in sheets of undifferentiated tissue. The question of dedifferentiation, according to Champy, can now be considered as settled, since Fischer[3] and Ebeling and Fischer[4] have proved that epithelium cultivated *in vitro*

[1] Champy, *Bibliog. Anat.*, 1913, xxxiii, 184.

[2] Drew, A. H., Three lectures on the cultivation of tissues and tumors *in vitro*, *Lancet*, 1923, i, 785, 833; A comparative study of normal and malignant tissues grown in artificial culture, *Brit. J. Exp. Path.*, 1922, iii, 20.

[3] Fischer, A., A three months old strain of epithelium, *J. Exp. Med.*, 1922, xxxv, 367.

[4] Ebeling, A. H., and Fischer, A., Mixed cultures of pure strains of fibroblasts and epithelial cells, *J. Exp. Med.*, 1922, xxxvi, 285.

does not dedifferentiate, but can be cultivated indefinitely and that it continues to behave morphologically and biochemically as epithelium. Drew[2] describes also in his paper a keratinization of epithelium in cultures which sets in when connective tissue cells are added and attributes it to a specific action of the connective tissue cells. The purpose of the experiments reported in this paper is to show that both the phenomena described by Drew are related and that as a rule they take place in the absence of fibroblasts or connective tissue cells.

In the paper by Ebeling and Fischer[4] can be found an illustration (Fig. 6) of epithelium in the 13th passage of a pure culture, growing in "finger-shaped" processes, similar to those in an illustration in the paper by Drew. There had never been the slightest contamination of my own culture with fibroblasts during the cultivation, yet, nevertheless, the epithelium differentiated in the sense of Drew, that is to say to primitive tubules. Fig. 1 of the present paper shows a pure culture of epithelium at the 18th passage, and one can see that the new growth consists only of fine tubules in spite of the fact that the cells had not been in contact with connective tissue cells for about 6 weeks.

The same can be said of the keratinization. Fig. 2 demonstrates that. The figure represents a section through a pure culture of epithelium in its 22nd passage. The entire central portion shows a beautiful keratinization. The culture was stained by Van Gieson's method and nowhere could any pink elements be detected in the tissue. Had there been any connective tissue present, it would certainly have attracted attention, as has been shown (Ebeling and Fischer).[4]

The organization of epithelial cells cultivated *in vitro*, occurs when the growth, for one or another reason, suffers and becomes irregular. It may result when the tissue is not properly placed in the culture medium—or more usually—when the central portion of the tissue becomes thick and more or less necrotic because of the poor supply of oxygen and of nourishment. According to my experience, the keratinization of epithelial cultures is always the result of lack of oxygen and nourishment. It cannot, of course, be denied that the presence of connective tissue cells, as described by Drew, can be responsible for the same result, but the fact should be emphasized

that both phenomena will occur in the absence of fibroblasts, and that the organizing or, according to Drew, the "differentiating" influence of connective tissue cells is in no sense specific. This does not detract from the interest of the observation that connective tissue cells cultivated side by side with epithelium bring about a quick organization and keratinization of the cells.

SUMMARY.

The organization, or, according to Drew, differentiation, of epithelial cells grown *in vitro* is not due to a specific influence of the associated connective tissue. It occurs in all old pure cultures of epithelium, when the tissue fragment becomes thick and suffers from lack of oxygen and nourishment. Under the same circumstances, keratinization occurs in cultures of epithelium.

EXPLANATION OF PLATE 29.

FIG. 1. Experiment 1309. 18th passage of a pure culture of epithelium 48 hours old, from the iris of the chicken embryos. The new growth is formed as rudimentary tubules.

FIG. 2. Experiment 1476. 22nd passage of a 48 hour old pure culture of epithelium from the iris of the chicken embryos. The photograph is taken of a section through the culture and the keratinization can be seen.

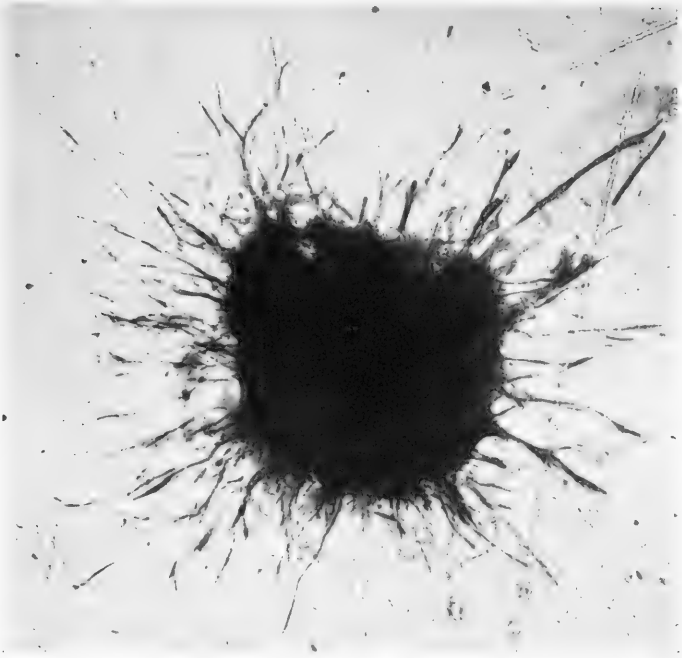


FIG. 1.

FIG. 2.

(Albert Fischer: Keratinization of epithelium *in vitro*.)

EXPERIMENTAL STUDIES WITH A SPIRAL ORGANISM FOUND IN A WILD RAT.

By H. MOOSER, M.D.

(*From the American Hospital, Mexico City, Mexico.*)

PLATES 30 to 32.

(Received for publication, November 9, 1923.)

The researches of the Japanese on infectious jaundice, seven-day fever and rat-bite fever have brought out the fact, that certain rodents harbor the causative agents of these diseases.

The present paper deals with a spiral organism isolated from a wild rat (*Mus decumanus*) in Mexico City.

The search for it was undertaken on account of the observation of two cases of severe illness in children bitten by rats.

Material.

I saw last year two cases of rat-bite fever with a typical fever course, swelling of the bitten fingers, lymphangitis, lymphadenitis, and a maculopapular exanthem. One was a Mexican girl who was bitten by a rat in the schoolyard. She found the animal lying in a corner and touched it. Apparently the animal was quite sick. The girl was ill for more than six months, but recovered. The second case was a American boy who was bitten by a wild rat which had entered his rabbit cage. He was sick with paroxysmal relapsing fever for 9 weeks. I was called in both cases to do a blood count as the attending physicians took the disease for a peculiar form of sepsis. I made the diagnosis rat-bite fever on account of the fever type and the peculiar exanthem. I found in both cases a moderate polynuclear leucocytosis and signs of a secondary anemia, but no spirochetes. Unfortunately in neither case was I allowed to draw blood from a vein. I decided to examine some wild rats in the hope of finding the microorganism described by the Japanese authors. In one out of the twenty rats I got for examination I found in the blood

a small motile spiral organism with a long flagellum at each end. The animal was killed and emulsions of the internal organs injected subcutaneously into six white rats.

Experiments with White Rats.

Blood from these white rats was examined daily with the dark field, but it was only after 3 weeks, that I found a few organisms resembling those in the original injection material. Blood was taken with a fine needle from the heart of one of the infected rats and injected into six young white rats. 3 days after the injection a few of the organisms were found in the blood of one young animal, 4 days later four were found to be positive and on the 6th day they were found in all. The number slowly increased in the blood for about a week and then slowly decreased again. After 4 weeks only an occasional one was found in the blood. The rats did not show any clinical signs of illness except a transitory swelling at the place where they were injected. Several rats were killed after they had been kept under observation for 4 or 5 weeks. There was a hyperemia of the internal organs with a very few old and fresh hemorrhagic spots in the lungs. With Levaditi's method I found, but only after repeated careful examinations, a few of the spiral organisms in the kidneys and adrenals. In one rat I found several specimens in the wall of a kidney vein. Experiments were now begun with guinea pigs.

Experiments with Guinea Pigs.

Blood was taken from the tail of a rat which was found to have the organisms in the blood and injected with a capillary tube into the scrotum or the labium majus of six guinea pigs. The injected parts became red and swollen within 3 to 5 days with a swelling of the regional lymph glands. The swelling subsided within 3 to 4 days only to light up again afterwards. Between 10 and 14 days after inoculation the organisms were found to be present in the blood by dark-field examination. The number gradually increased for a week to decrease slowly until the death of the animals which occurred between the 26th and the 31st days after inoculation. When the number of organisms was at the highest, there were never more than

eight to ten, in one microscopical field. Although they decreased in number they were found always by direct observation with the dark-field microscope or in stained films (Fig. 3). Between the 15th and 20th day a grayish cloudiness in the form of a ring was found in both corneas of one guinea pig, somewhat resembling an arcus senilis. In a few days the whole cornea became milky and fine vessels entered from the periphery of the cornea. This keratitis cleared up in a few days but was soon followed by a relapse. The conjunctiva became injected and the soft parts surrounding the eye became swollen. The conjunctiva discharged a mucopurulent secretion in which by careful examination a very few motile spiral organisms were found. Dr. Bauer, our hospital oculist, confirmed my diagnosis of parenchymatous keratitis. This keratitis and conjuntivitis appeared in all the infected guinea pigs and was in later experiments found to be a very constant occurrence (Figs. 1 and 2). The hair around the eyes began to fall out, the eyelids became thick and infiltrated, and showed many rhagades; also the hair around the nostrils started to fall out. The guinea pigs now began to lose weight rapidly; there was an extreme progressive atrophy of the whole muscular integument and they screamed when handled as if they had intense pain. Walking became difficult for them; the hind legs especially were stiff and helpless. They died in a comatose condition. Films made with the blood showed marked anisocytosis, polychromatophilia, and nucleated red cells. During the course of the infection all animals had irregular intermittent fever and a paroxysmal relapse was often accompanied by a new cloudiness of one or both corneæ and a new swelling of the primary lesion.

Postmortem Findings.—Extreme emaciation; subcutaneous connective tissue dry, with only a little brownish, gelatinous, fatty tissue. Whole muscular integument atrophic and dry. Some lymph glands, especially those in the mediastinum injected and quite enlarged. Lungs only slightly collapsed, the substance of the lungs somewhat indurated, more or less brownish in color with old and fresh hemorrhagic spots. Heart slightly hypertrophic, myocardium somewhat translucent, endocardium apparently normal. Liver large, congested, somewhat opaque. Spleen dark, hyperemic, enlarged. Adrenals enlarged, with a few fresh and old hemorrhagic spots (not found in all animals). Kidneys hyperemic, showing in two animals a few small irregular contracted scars on the surface. In one of the guinea pigs these scars were found to be quite pronounced, the kidneys having the appear-

ance of a beginning arteriosclerotic kidney. The capsule in this case was quite adherent. The parenchyma of all the kidneys showed always more or less cloudy swelling of the cortex and the boundary between cortex and medulla was not sharp. In the columnæ Bertini fine yellowish spots were to be seen now and then. The whole organ, especially the cortex was pronouncedly hyperemic. Intestines, brain, medula, testicles, and ovaries were apparently normal.

Microscopical Findings. 1. Cornea.—Tissue edematous, connective tissue cells enlarged and increased in number. Between the fibers were polynuclear leucocytes, few lymphocytes, and plasma cells. On the limbus of the cornea there was a dense infiltration with plasma cells and lymphocytes around young vessels, and capillaries entering the substance of the cornea (Fig. 4). Epithelia of the cornea were swollen and the nuclei enlarged. A few mitotic figures were to be found and some polynuclear wandering cells between the epithelia.

2. Eyelids.—Edema and dense infiltration of the mucous membrane with lymphocytes, leucocytes, and plasma cells. The same condition existed in the skin of the eyelids.

3. Lacrimal Glands.—A slight infiltration of the stroma and ducts with lymphocytes and leucocytes was found in all animals.

4. Brain.—Tissue hyperemic and somewhat edematous. In all the animals I found small but very distinct proliferations of neuroglia cells around capillaries in the gray matter just beneath the layer of the large pyramidal cells (Fig. 7). Occasionally such nodules were found in the basal ganglia in the gray matter of the hippocampus and in the cerebellum. They resemble closely the *Gliaherde* found by Duerck in the brain of individuals that had been comatose from malaria. I found in the cerebellum of one animal changes in the molecular layer resembling closely Spielmeyer's *Gliastrauchwerk.*

5. Heart.—The organ proved normal in one animal but in five others there was some cloudy swelling of the muscular fibers but no fatty infiltration. The interstitial tissue increased, and in one animal densely infiltrated with lymphocytes and fibroblasts especially beneath the endocardium.

6. Lungs.—A difference was here to be noted between the animals which died on the 26th day and the animal which died on the 31st day. This difference was one of degree, one lung showing early, the other, advanced changes. The early change was represented by extreme hyperemia of the whole parenchyma of the lung. The capillaries were greatly distended showing a great number of polynuclear leucocytes in the lumen. The septa were enlarged containing polynuclear leucocytes and young fibroblasts which held hemosiderin. The alveolar epithelia were swollen and a slight degree of desquamation was sometimes found, but no pneumonic foci. In the interlobular septa fresh and old hemorrhages were found, and very exceptionally red cells were to be seen in the alveoli. There was a dense infiltration with lymphocytes, plasma cells, and fibroblasts around the small vessels. In the bronchi a slight catarrhal condition was present with desquamation into the lumen. This condition more or less pronounced occurred in five guinea pigs. In the animal which succumbed on the 31st day the microscopic picture resembled, some-

what, the interstitial form of a pneumonia alba. The septa were much enlarged and in some places no alveoli were seen. This disappearance of the alveoli was not due to a hepatization but to a collapse or a compression of the alveolar apparatus by connective tissue very rich in cells, as could be demonstrated through Weigert's coloration for elastic fibers. It consisted of fibroblasts, with comparatively few leucocytes, lymphocytes, and plasma cells. Many of the fibroblasts were densely filled with hemosiderin. Around the small vessels and bronchi there was still a dense infiltration with wandering cells and a pronounced catarrhal condition of the smaller bronchi was present. Endarteritis obliterans was found in a few small arteries. The capillaries and small vessels were distended and hyperemic, containing a few bone marrow giant cells; but not solely on account of compression had the alveolar structure disappeared in many parts of the lung. With Weigert's method an organization of the alveolar apparatus by fibroblasts and desquamated alveolar epithelia containing hemosiderin was found (Fig. 5). Occasionally the fibroblasts were observed to have formed submiliary nodules with radiary arrangement like epithelioid cells. In one instance I saw such a nodule with central necrosis surrounded by giant cells of the Langhans type. The elastic fibers in such organized parts were markedly decreased and their architectonic structure split up.

7. Liver.—The findings in the liver were more uniform. Some cloudy swelling of the liver cells existed within the periphery of the acini hyaline droplets and occasionally fine fat droplets. The stroma was somewhat increased by young connective tissue which showed a moderate infiltration with lymphocytes and leucocytes. Around small branches of the portal vein there was an occasional denser infiltration.

8. Kidneys.—The changes in the kidneys were found to be predominantly interstitial as in the case of the lungs. There was at their beginning a pronounced hyperemia, principally in the cortex. The glomeruli were hyperemic with many polynuclear leucocytes in the capillaries. Some swelling of the epithelia of the tubuli contorti was to be noted and a lesser one of the tubuli recti. An insignificant fatty infiltration existed in the cells of a few tubules with desquamation and desintegration of the epithelia and hyaline cast formation. The retractions in the cortex mentioned in the postmortem findings were formed by scars in which the glomeruli and tubes were atrophic, and some glomeruli completely hyaline and embedded in hyaline connective tissue (Fig. 6). Apparently this represented the final stage in the process which began with a dense infiltration of the interstitial tissue beneath the capsule by polynuclear leucocytes, pseudo-eosinophils, plasma cells, lymphocytes, together with an extravasation of red cells as seen in other parts of the kidneys. Desquamation and proliferation of the parietal part of Bowman's capsule and also hyaline exudate into the capsule were occasionally found. The blood vessels appeared normal except that a moderate swelling of the intima of the vas afferens was occasionally seen. Hemosiderin was found here but only in a few cells near the kidney surface.

9. Adrenals.—Pronounced hyperemia existed especially in the cortex. Occasionally small hemorrhages were present beneath the capsule and some infiltration of the stroma with lymphocytes.

10. Spleen.—This organ showed a marked increase of polynuclear leucocytes mostly eosinophils. A great number of myelocytes and nucleated red cells were found in the spleen of two animals. Hemorrhages and small hemorrhagic infarcts were present in three animals. A good deal of hemosiderin was found in the stroma.

11. Pancreas.—Infiltration of the stroma with lymphocytes and leucocytes was seen in one animal.

12. Spinal Cord.—Not examined.

13. Peripheral Nerves.—No lesions found.

14. Primary Lesions.—This showed a pronounced edema of the connective tissue together with a dense infiltration by leucocytes, lymphocytes, and plasma cells and some desquamation and infiltration of the lymph vessels. There were infiltration and necrotization of the epidermis.

15. Lymph Glands.—Hyperplastic condition of the follicles existed with desquamation and infiltration of the sinuses by leucocytes and lymphocytes.

16. Thyroid Gland.—Atrophic, no gross changes.

17. Salivary Gland.—Some infiltration of the stroma and ducts with lymphocytes and leucocytes. Desquamation of the epithelia of the gland ducts in three animals.

18. Testicles and Ovaries.—Normal.

19. Skeletal Muscles.—There was a pronounced atrophy of the fibers with proliferation of the nuclei of the sarcolemma. Some fibers showed complete loss of the striation and hyaline, and seldom a waxy degeneration. Infiltration of the interstitial tissue with lymphocytes and eosinophilic leucocytes was also present.

20. Bone Marrow.—A marked increase of myelocytes, megalocaryocytes, and nucleated red cells was seen in three animals.

The Organism.

An accurate study of the morphology of the living organism was difficult on account of its very rapid bipolar progression, especially in the blood drop. In secretion from the eyes the movements were not as rapid. The best material for examination was a drop of serum squeezed from the primary lesion after puncture with a fine needle. The organisms were found here in enormous numbers.

With the dark field a fine flagellum was always observed at each end of the body. It was seen as a straight or finely spiral filament. The body measured about 2 to 4 micra in length and specimens from tissue occasionally 5 to 6 micra. The elementary spirals were rigid, regular, and constant in number, measuring about 1 micron in length. Spiral depth 0.6 to 0.7 micron in the center of the body. The body seemed to be round and measured about 0.3 micron in thickness. It was slightly tapered at both ends and the spiral amplitude also decreased somewhat

towards the extremities. Bending of the body was observed. In larger organisms a shallow wave was often seen. I observed three kinds of progressive movement in the dark field: (1) in small individuals which moved very rapidly through the optical field both flagella were seen as straight, motionless filaments in the axial line of the body; (2) a fine very refractile button was seen at the tip of the straight rear flagellum which apparently was the optical effect of the rapid small excursions of the tip; (3) in larger individuals the flagellum was occasionally seen to make undulatory movements and the body deviations from the axial line like a vibrio.

The explanation of the first kind of movement caused me some difficulty on account of the very rapid progression of the organism, the body of which was quite transparent under the dark field. One specimen pushing against a blood platelet furnished me the explanation. It could not move further; the body was seen in rapid spiral movement, the rear flagellum making vigorous large excursions. By slight pressure on the cover-glass the blood platelet was moved aside and the spiral organism now progressed with great rapidity along the field with a straight motionless flagellum at the back end, like a fish which with one rapid stroke of the tail propels itself a great distance. Occasionally the front flagellum was seen bent back along the body of a quickly moving specimen. The rear and front flagella sometimes steered the direction of the movement by quick bending. The flagella measured 2 to 3 micra. Small specimens seemed to have proportionally longer flagella. Living and stained specimens were seen in transversal division. Once I found three individuals arising from one.

I was not able to see more than one flagellum at each end of the organism. In a well lighted dark field I saw *always* one at each end. The organisms stained with Giemsa's solution, Leishman's stain, ordinary aniline dyes and were easily demonstrated with the method of Levaditi and Fontana. Staining of the flagella was only occasionally achieved but they could be easily demonstrated by Burri's method, not so well by Fontana's. Also with these methods the flagella were generally seen as straight or very finely spiral filaments. In living specimens the flagella were probably always in spiral form. In movement they were readily seen in the dark field and gave the impression of a refractile mass, but in stained specimens they could hardly be observed. The organisms are destroyed by 10 per cent saponin, sodium taurocholate, and sodium glycocholate.

I found in the literature only one organism which in form and movement has the same characteristics as that here described, namely *Spirochæta morsus muris* of the Japanese authors. Futaki and coworkers think that their organism is a spirochete belonging to the *Genus treponema*. Adachi succeeded by a special method in staining several flagella at each end of the body of the spiral organism of rat-bite fever and calls it a spirillum. I was not able to demonstrate several flagella in my organism. The fact that I observed bending

of the body indicates that I am dealing with a spirochete. The very constant number of the spirals and their rigidity is characteristic for the *Genus treponema*. The difference between this organism and the treponemata that are well known lies principally in the very rapid movement of the former which is due to the presence of large vigorous flagella. Noguchi denies the presence of true flagella in this whole group of microorganism. The movement of a spironema and a leptospira is comprehensible without the presence of a flagellum. The progression accompanied by axial rotation of a short specimen of a treponema however, in which only occasionally a transient shallow wave may be observed, cannot be explained without motive power in form of a flagellum. The difference between the flagellum of the organism described and the "terminal finely spiral filament" of Noguchi is not an essential one. I classify, therefore, the microorganism I isolated from a wild rat under the *Genus treponema*.

Experiment 1. Transmission Experiments with Biting Guinea Pigs.—A strong male guinea pig 520 gm. in weight, known to me as a fighter was infected with a drop of blood from an infected guinea pig. Spirochetes were found in the blood on the 10th day after inoculation. The same day the animal was placed with a non-pregnant female and another strong male of 460 gm. An hour later the smaller male had a deep bite on a lip. It was then separated for observation and examined daily. The wound healed within a few days without swelling of the surrounding tissues and the animal had no fever. 20 days later it was in good condition and no spirochetes were found in the blood. It was killed 28 days after it was bitten and the examination of the internal organs did not show any pathological changes. On the 17th day after inoculation the larger male showed cloudiness of one cornea and swelling and discharge from both conjunctivæ. It was put with a young male and bit it several times. This animal later developed typical symptoms of the infection and the spiral organisms were easily demonstrated in the blood 11 days after it was bitten.

Experiment 2. Transmission Experiments with Secretion from the Eyes.—A guinea pig was used which showed well developed keratitis and conjunctivitis. With a very fine capillary tube a small quantity of secretion was aspirated from the conjunctival sack and injected

into the labium majus of two guinea pigs. Special care was taken not to produce any lesion of the conjunctiva or of the cornea of the animal while procuring the material. 5 days after the injection the labia were found to be very swollen and appeared to be painful. No spirochetes were found in the blood, but the guinea pigs had fever (101.8°–102°F.) on that day. In a drop of aspirated serum from a swollen part I found a few spirochetes with the dark field. From the 10th and 11th days spirochetes were found in the peripheral blood and the guinea pigs both succumbed later with the typical symptoms.

Experiments with Salvarsan.

Two guinea pigs which had numerous spirochetes in the blood were injected intravenously with neosalvarsan. The next day no spirochetes could be found in the blood, but after 8 to 14 days spirochetes were found in both and the guinea pigs died with the usual symptoms.

Eye Lesions in Rats.

After the finding of living spirochetes in the discharge from the eyes of the guinea pigs and after the positive transmission experiments with this discharge I examined carefully all my white rats which had been infected 4 and 5 months before. The total number of infected rats was twenty-six. Eight of them now showed conjunctivitis, only four had conjunctivitis, keratitis, and iritis with synechia; four, a shrinkage of one or both eyes with iritis; and one, a fresh keratitis. The remaining nine animals were apparently normal.

Dark-field examinations of the secretion from the eyes of these rats resulted negatively. To find out if the eye lesions in rats play a part in the transmission of the disease, the following experiment was carried out. Previous to it I had allowed the rats to bite on wood, toast, and even on iron in order to see whether this would give rise to abrasions. No blood could be found afterwards in their mouths.

Experiment 3. Transmission Experiment with Blood and Discharge from the Eyes of a Rat Infected 4 Months Before.—Four young

guinea pigs were used. A rat was chosen which showed iritis and conjunctivitis and Guinea Pigs 1 and 2 were injected subcutaneously with a drop of discharge of the eye taken with a capillary glass tube, while Nos. 3 and 4 were inoculated with a drop of blood from the rat. Nos. 1 and 2 developed infection with the spiral organism, whereas Nos. 3 and 4 remained well.

Distribution of the Organism.

As already mentioned the organisms were always found in the blood of the guinea pigs, although they decreased markedly in number prior to death of the animals. They could be found with Levaditi's method in every organ by repeated and careful examination but never in great number. In the kidneys and adrenals they were found with greater ease than anywhere else, save in the cornea. Here I found them without difficulty in all six animals and nearly always in great numbers, especially when the keratitis was not very pronounced. When the keratitis was advanced and resorption and organization of the inflammatory exudate by blood vessels had taken place, they were rather scarce, doubtless as the result of an induced immunity. As the normal cornea has no blood vessels the organisms may be supposed to have multiplied there unaffected by circulating antibodies at a time when they are already scarce in the blood stream and in the viscera.

There was another finding most readily to be explained by the development of immunity in the infected animals. While in the central parts of the cornea small, regular organisms were found, in the periphery, where entering blood vessels and dense infiltration with wandering cells appeared, thick, huge, irregular forms such as Kaneko and Okuda have pictured, were met with. The organisms were more numerous at the periphery of the cornea and in the layers near the surface epithelium. Once I found one between the epithelial cells. As already mentioned I found them living in the conjunctival sack and there can be no doubt, that they had passed the cornea.

DISCUSSION.

When I found the spiral organism in a *Mus decumanus* in Mexico City and transmitted it successfully to white rats and guinea pigs, I was convinced, that I was dealing with the spirochete known from the publications of the Japanese workers as *Spirochæta morsus muris*. My organism presents the same morphological aspect and is characterized by the same swift rapid movements. It likewise kills guinea pigs invariably in a comparatively short time and produces inflamation of the injected part after between 3 and 5 days with a swelling of the regional lymph glands. Also it causes the same irregular intermittent fever, has the same low virulence for white rats, increasing in their blood for a short time to decrease slowly again, without producing clinical symptoms, though it could be recovered again after many weeks and months from the blood or viscera. But in spite of all this and the observation of two typical cases of rat-bite fever in Mexico City, I am not at present able to state positively, that I have been working with *Spirochæta morsus muris* because of the lack of precise tests for the identification of the latter. A comparison of the pathological findings in my guinea pigs with those in human rat-bite fever speaks strongly, however, in favor of the view that both are caused by the same organism.

Blake has found acute ulcerative endocarditis, subacute myocarditis, interstitial nephritis, glomerular and interstitial hepatitis, and hemorrhagic infarct of the spleen in the human subject. With the exception of the endocarditis I found the same changes to be more or less pronounced in all my guinea pigs. Kaneko and Okuda found in their first human case interstitial and glomerular nephritis as well, but they assume that this nephritis existed before the patient became ill with rat-bite fever. The gross changes in the organs of Blake's case they attribute to the infection with a streptothrix which he isolated from his case. But when one considers the changes in my guinea pigs it seems possible that the rat-bite spirochete played a prominent part in the pathology of both cases.

Manson reports the frequent occurrence of keratitis in human rat-bite fever and the finding of the spirochete in the eye. The mode of infection in rat-bite fever is not evident from the work of the Japanese authors. Since they never found the spirochetes in the mouth of infected rats, they assumed that transmission must take place as result of hemorrhage from the gums of the biting animals. I think that the experiments reported in the present paper give a more reasonable explanation of the transmission of the spirochetes. These indicate a direct relationship

between the occurrence of eye lesions and transmission through bites. The absence of such lesions would explain why I did not contract the disease in spite of several bites I received from infected rats. Experiment 3 shows clearly that hemorrhages from the gums of a biting animal is an unlikely method of transmission. The two guinea pigs inoculated with rat blood did not get sick, whereas the two others injected with discharge from the eyes of the same rat contracted the disease. Experiments 1 and 2 yield further evidence in the matter. The spiral microorganism passes through the lacrimal channel into the nasal cavity and thence may enter the mouth though its presence there is not absolutely necessary for transmission through bites. For the upper lip of the rats and guinea pigs is cleft to the nostrils and since the biting animal touches the bitten object with the nose, the organism may enter the wound from this point. I found in three guinea pigs slight changes in the salivary glands, and it is possible, that occasionally a spirochete may enter the mouth by this route.

SUMMARY.

1. Two cases of rat-bite fever with a typical fever course and a maculopapular exanthem came under my observation in Mexico.

2. A spiral organism morphologically identical with one known in Japan as the causative agent of Sodoku was found in a *Mus decumanus* caught in Mexico City.

3. The organism was found to be pathogenic for white rats and guinea pigs, the infection being fatal for guinea pigs.

4. The bite of an infected guinea pig led in one of two instances to infection and death of the animal bitten.

5. The microscopical findings in the guinea pigs infected with this microorganism closely resemble in many respects the findings in Blake's case and in Kaneko and Okuda's first case, the only ones of human rat-bite fever which have been closely studied at post mortem.

6. Eye lesions were found in all the infected guinea pigs and the spiral organisms were demonstrated in the discharge from the eyes and also within the cornea.

7. Similar eye lesions were found in most of the infected rats and the secretion from the eyes of one of them was demonstrated to be infectious for guinea pigs.

8. The eye lesions constitute an evident source for the spiral organisms transmitted by biting, and not improbably the only one.

My experiments may give an explanation for the transmission of some other spirochetal diseases besides rat-bite fever. Cases of Weil's diseases are reported to be caused by the bite of a rat and it is noteworthy that eye lesions occur in spirochætosis icterohæmorrhagica (Manson).

I am greatly indebted to Dr. P. W. Monroe for reading the manuscript, to Prof. Mesa Gutierrez for making the photograph of the blood slide, and to Dr. C. Bauer for examining the eyes of the animals.

BIBLIOGRAPHY.

Adachi, K., *J. Exp. Med.*, 1921, xxxiii, 647.
Blake, F. G., *J. Exp. Med.*, 1916, xxiii, 39. (Blake's original article was not available.)
Duerck, H., quoted by Oesterlin, E., *Z. ges. Neurol. u. Psychiat.*, 1920, lvii, 34.
Futaki, K., Takaki, I., Taniguchi, T., and Osumi, S., *J. Exp. Med.*, 1917, xxv, 33.
Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1916, xxiv, 471; 1917, xxvi, 341.
Ido, Y., Ito, H., and Wani, H., *J. Exp. Med.*, 1918, xxviii, 435; 1919, xxix, 199.
Ido, Y., Ito, H., Wani, H., and Okuda, K., *J. Exp. Med.*, 1917, xxvi, 377.
Inada, R., Ido, Y., Hoki, R., Ito, H., and Wani, H., *J. Exp. Med.*, 1916, xxiv, 485.
Ishiwara, K., Ohtawara, T., and Tamura, K., *J. Exp. Med.*, 1917, xxv, 45.
Kaneko, R., and Okuda, R., *J. Exp. Med.*, 1917, xxvi, 325, 363.
McFarland, J., A text-book on pathogenic bacteria and protozoa, Philadelphia, 9th edition, 1919.
Manson, P., Tropical diseases, New York, 8th edition, 1923.
Noguchi, H., *J. Exp. Med.*, 1917, xxv, 755.
Spielmeyer, W, quoted by Oesterlin, E, *Z. ges. Neurol. u. Psychiat.*, 1920, lvii, 34.
Spielmeyer, W., *Münch. med. Woch.*, 1919, lxvi, 313.

EXPLANATION OF PLATES.

PLATE 30.

FIG. 1. Guinea pig. 20 days after inoculation with a drop of blood of a white rat. Pronounced keratitis and conjunctivits. Marked hair fall and thickening of the eyelids.

FIG. 2. Same animal. Falling of the hair on the nose.

FIG. 3. Smear of the peripheral blood of an infected guinea pig, showing the organism, Leishman's stain.

PLATE 31.

FIG. 4. Section of the cornea of an infected guinea pig. Entering blood vessel surrounded by lymphocytes and plasma cells. Hemalum-eosin, Zeiss obj. D.D.

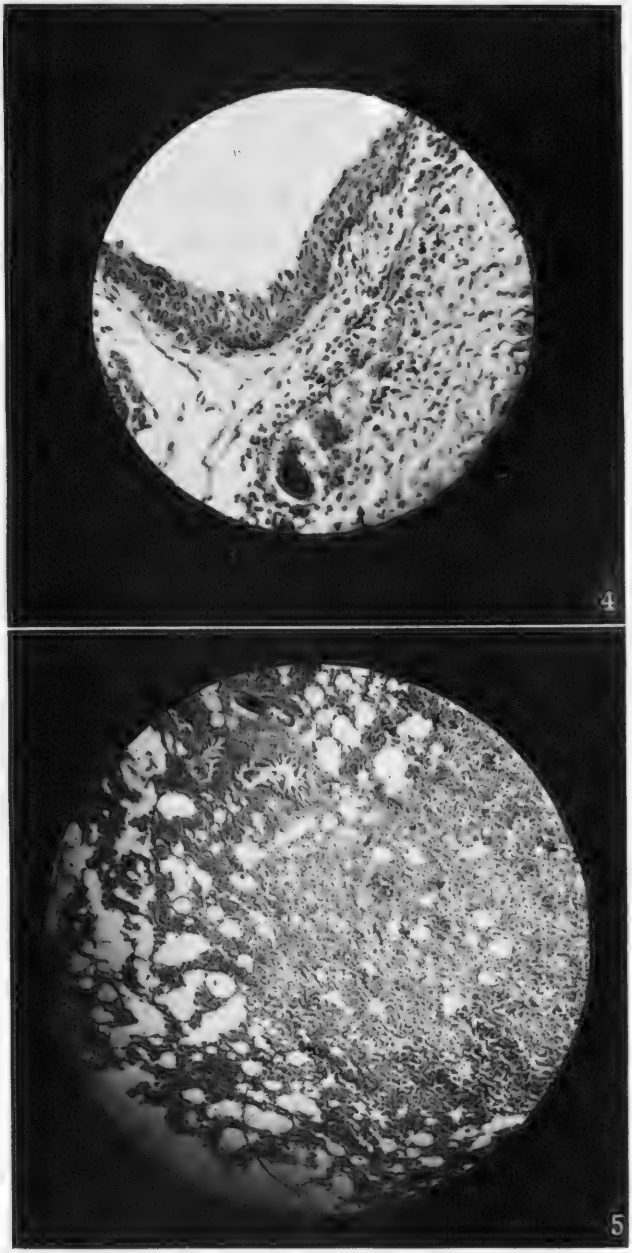
FIG. 5. Lung thickening of the interstitial tissue, atelectasis, and organization of the alveoli with compensatory emphysema. Hemalum-eosin Zeiss obj. A.

PLATE 32.

FIG. 6. Kidney. Zeiss obj. D. D.

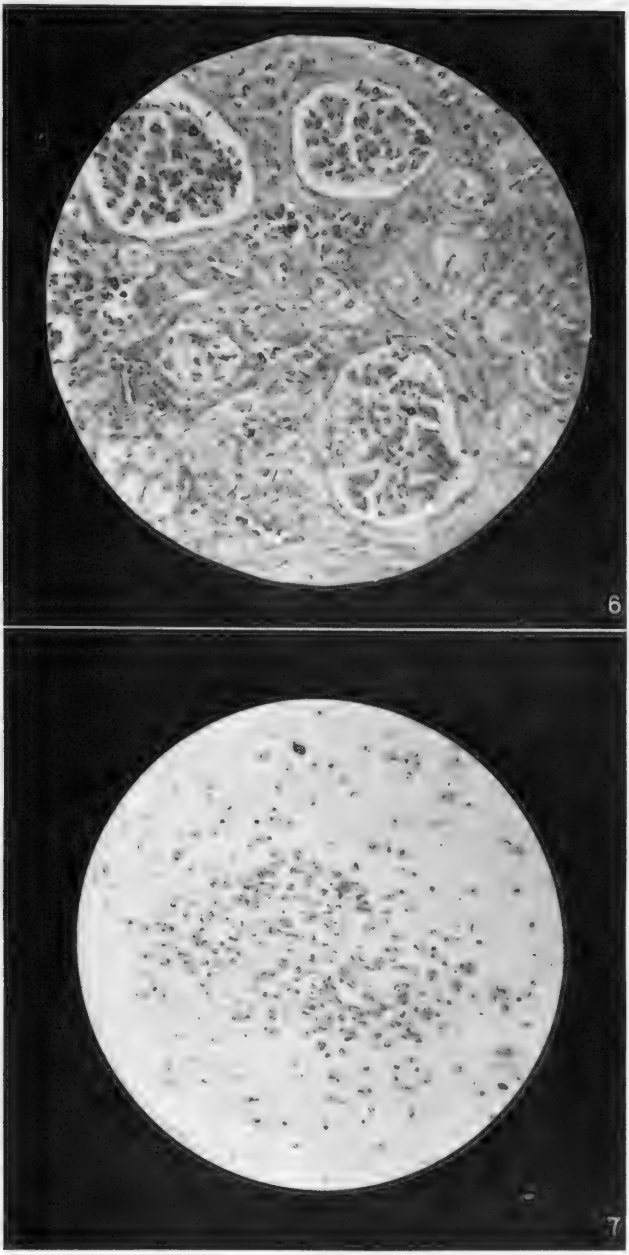
FIG. 7. Brain. Thalamus opticus. Glianodule. Toluidine blue. Zeiss obj. D.D.

(Mooser: Spiral organism in wild rat.)



(Mooser: Spira lorganism in wild rat.)

UNIV. OF MICH.



(Mooser: Spiral organism in wild rat.)

OF MICH.

THE RATE OF HEALING OF WOUNDS.

By JOHN BROWNLEE, M.D.

(*From the National Institute for Medical Research, London, England.*)

(Received for publication, November 2, 1923.)

During recent years a number of papers by Carrel and others concerning the rate of healing of wounds have been published. Du Noüy has also applied some mathematical treatment to these observations and constructed some graduation formulæ. In the application of these formulæ, however, I think that the meaning of the figures has been in some measure obscured and that a different treatment reveals some interesting relations.

Personally I do not feel inclined to make a special formula for any individual case until those which occur so commonly in nature have been investigated. The formula which seems to apply in all cases in which the data are sufficient is the geometrical progression. A geometrical progression is found to arise in nature in many different ways from the theory of chance. It might be assumed that at the beginning of the healing of a wound, the impulse to heal is great and the factors on which this impulse to heal depends, decay according to the ordinary monomolecular reaction, but the geometrical progression turns up in so many different ways and for reasons which at present we cannot understand that this is nothing but a tentative suggestion. To say more is going beyond the evidence.

To clear the ground, a further point requires mention; namely, that it is not necessary to assume that the process of healing is continuous and not subject to sudden change. Take the recent work of Glenny and Hopkins[1] on the disappearance of diphtheria antitoxin from the blood of animals into which intravenous injections have been made. In these experiments it is found that the rate of disappearance of the antitoxin is not continuous. During the

[1] Glenny, A. T., and Hopkins, B. E., *J. Hyg.*, 1922–23, xxi, 144.

first day and a half after injection a very large quantity of antitoxin disappears. Thereafter the decrease is much more gradual and obeys the law of the monomolecular reaction, the amount decreasing in the terms of a geometrical progression. After an incubation period of 9 or 10 days has passed, when antibodies begin to form, the destruction of the antitoxin becomes suddenly much more rapid. There is no use in trying to fit a continuous curve to a case like this in which, from the nature of the change which takes place, a fundamental break in the physiological or pathological processes is introduced.

When the figures published by Carrel are examined, something similar to this will I think be at once evident.

In the second column of Table I, the figures relating to the healing of a wound in the thigh, in a male aged 38 years are shown, the measurements of the area of the wound being given at intervals of 4 days. In the fourth and fifth columns, the logarithms of these numbers are given and the differences between the successive values of the logarithms. From the figures it would appear that at a date between February 29 and March 4 healing began. It will be noticed that from this date up to March 24, the mean difference of the successive logarithms is about 0.1, whereas following March 24 it is much nearer 0.2. By graduating the observations to two geometrical progressions with a break at about March 24, the figures in the third column have been obtained. The fit can only be considered exceedingly good. The ratio in the first half of the observation period from 4 days to 4 days is 0.793 and in the second half 0.652, the second value being very nearly the square of the first.

If the preceding case were a solitary one it might be considered as due to chance, but the same phenomenon is seen in practically every case given by Carrel. Two more examples are shown in Tables II and III and a third in Table VII.

In an experimental case of shorter duration, a female dog of 13 years of age had a rectangular flap of skin excised from the dorsal region. For 6 days no sign of reaction appeared, when, as is the rule in these cases, a rapid healing began. From the time healing began, about May 18, until June 9, the process can be graduated

very closely to a geometrical progression with a ratio of 0.785. This healing went on uninterruptedly until June 9 when again a sudden change took place and a wound which was 2.7 cm. was practically healed in 10 days. The area of the wound which would

TABLE I.

Male, Age 38 Years. Wound of the Thigh.

Date.	Area of wound.	Theoretical* area.	Ratio.	Log.	Difference.
1916	*sq. cm.*	*sq. cm.*			
Feb. 29	46.7	51.4		1.669	
Mar. 4	40.5	40.8		1.608	0.115
" 8	31.1	32.3		1.493	0.062
" 12	27.0	25.6	0.793	1.431	0.107
" 16	21.1	20.4		1.324	0.120
" 20	16.0	16.1		1.204	0.107
" 24 ⟶	12.5	12.8		1.097	0.178
" 28	8.3	8.3		0.919	0.220
Apr. 1	5.0	5.4		0.699	0.097
" 5	4.0	3.5	0.652	0.602	0.240
" 9	2.3	2.3		0.362	0.216
" 13	1.4	1.5		0.146	0.146
" 17	1.0	0.8		0.000	

The data for this table were taken from du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 465.

* Healing would begin by this theory on Mar. 2.

have been present had the process remained continuous is given in the third column. In comparison with this two other short cases are given in Tables V and VI, in both of which the graduation to a geometrical progression is nearly perfect and in both of which towards the end a rapid quickening in the rate takes place.

TABLE II.

Male, Age 32 Years.

Date.	Area of wound.	Theoretical area.	Ratio.	Log.	Difference.
1916	*sq. cm.*	*sq. cm.*			
Feb. 6	56.6	57.5		2.753	
					0.107
" 10	44.3	48.2		2.646	
					0.023
" 14	42.0	40.5		2.623	
					0.097
" 18	33.6	34.0		2.526	
					0.046
" 22	30.2	28.4		2.480	
			0.839		0.096
" 26	24.2	23.9		2.384	
					0.040
Mar. 1	22.1	20.0		2.344	
					0.114
" 5	17.0	16.8		2.230	
					0.096
" 9	13.6	14.1		2.134	
					0.093
" 13	11.0	11.8		2.041	
					0.077
" 17 ⟶	9.2	9.8		1.964	
					0.151
" 21	6.5	6.4		1.813	
					0.190
" 25	4.2	4.2		1.623	
					0.132
" 29	3.1	2.8		1.491	
			0.660		0.236
Apr. 2	1.8	1.8		1.255	
					0.194
" 6	1.15	1.2		1.061	
					0.158
" 10	0.8	0.8		0.903	

The data for this table were taken from du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 464.

The interpretation of these phenomena is conjectural. The first phenomenon is the sudden commencement of healing; this is so obvious that it cannot be overlooked. There seems, however, to

TABLE III.

Patient, Age 21 Years. Shell Wound of the Posterior Thoracic Region.

Date.	Area of wound.	Theoretical area.	Ratio.
	sq. cm.	*sq. cm.*	
Feb. 2	40.2		
" 6	40.0	38.3	
" 10	29.3	30.7	
" 14	24.0	24.6	0.788
" 16	19.8	19.6	
" 18	16.0	15.7	
" 22	11.0	10.8	0.675
" 26	7.1	7.3	
May 1	5.0	4.9	
" 5	2.0		

The data for this table were taken from Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, Text-fig. 15 between pp. 444 and 445.

TABLE IV.

Female Dog, Age 13 Years. Resection of a Rectangular Flap of Skin on the Dorsal Region.

Date.	Area of wound.	Theoretical area.	Ratio.
1919	*sq. cm.*	*sq. cm.*	
May 13	39.4		
" 17	38.7	43.3*	
" 19	33.5	34.0	
" 20	30.2	30.2	
" 23	20.3	20.9	
" 24	18.7	18.6	
" 26	14.3	14.6	
" 27	12.2	12.9	0.785
" 28	11.5	11.5	
" 29	9.2	10.2	
" 31	7.7	8.0	
June 4	4.0	4.9	
" 9	2.7	2.7	
" 13	1.1	2.1	
" 17	0.5	1.6	

The data for this table were taken from Carrel, A., and du Noüy, P. L., *J. Exp. Med.*, 1921, xxxiv, 341.

* Healing would begin on May 18.

TABLE V.

Patient, Age 36 years. Wound of the Left Iliac Region; Slightly Infected.

Date.	Area of wound.	Theoretical area.	Ratio.
	sq. cm.	*sq. cm.*	
Nov. 30	15.3	15.8	
Dec. 4	11.0	11.0	
" 8	7.5	7.7	
" 12	5.8	5.4	0.697
" 16	3.8	3.7	
" 20	2.7	2.6	
" 24 →	1.7	1.8	
" 28	0.7	1.2	

The data for this table were taken from Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 433.

TABLE VI.

Male, Age 38 Years. Wound of the Arm.

Date.	Area of wound.	Theoretical area.	Ratio.
1916	*sq. cm.*	*sq. cm.*	
Mar. 12	25.8	28.3*	
" 16	21.1	21.3	
" 20	16.0	16.0	
" 24	13.0	12.0	
" 28	9.9	9.0	
Apr. 1	6.5	6.7	0.750
" 5	5.6	5.1	
" 9	3.6	3.8	
" 13	2.7	2.9	
" 17	2.0	2.1	
" 21 →	1.7	1.6	
" 25	0.9	1.2	
" 29	0.5	0.9	
May 3	0.3	0.7	

The data for this table were taken from du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 466.

* Healing would begin Mar. 13.

TABLE VII.

Patient, Age 22 Years.

Date.	Area of wound.	Theoretical area.	Ratio.	Log.	Difference.
1916	*sq. cm.*	*sq. cm.*			
Feb. 6	129.4	171.8		2.112	
					0.067
" 10	111.0	137.4		2.045	
					0.022
" 14	105.5	109.9		2.023	
					0.025
" 18	99.5	87.7		1.998	
					0.169
" 22	67.5	70.0		1.829	
					0.073
" 26	57.0	55.9		1.756	
					0.103
Mar. 1	45.0	44.7		1.653	
					0.085
" 5	37.0	35.7		1.568	
					0.129
" 9	27.5	28.4		1.439	
			0.789		0.061
" 13	23.9	22.7		1.378	
					0.132
" 17	17.6	18.1		1.246	
					0.100
" 21	14.0	14.5		1.146	
					0.074
" 25	11.8	11.6		1.072	
					0.108
" 29	9.2	8.8	9.2	0.964	
					0.193
Apr. 2	5.9	6.1		0.771	
					0.158
" 6	4.1	4.2		0.613	
					0.136
" 10	3.0	3.0		0.477	
			0.695		0.247
" 14	1.7	2.0		0.230	
					−0.025
" 18	1.8	1.4		0.255	
					0.255
" 22	1.0	1.0		0.000	
					0.187
" 26	0.65	0.7		$\bar{1}$.813	

The data for this table were taken from du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 457.

be a second phenomenon. A point comes in the healing when an extra healing power suddenly develops. This change in the process seems to show that in surgical disease as in medical, there are more factors at work than might be thought probable. It is possible that a search into the conditions would furnish results of practical value.

A final note may be made on the case of the male, aged 22 years, in whom septic infection of the wound occurred and decreased the rate of healing (Table VII). After sterilization of the wound on February 22 and until March 29, healing progressed uninterruptedly with a ratio of 0.789. About March 29 a marked change took place, the ratio for the rest of the healing being 0.695. Du Noüy in fitting his formula obtained a curve which represents very closely the observations before and after sepsis and makes the inference that the sepsis had no permanent effect on the rate of healing. I have calculated back from the first portion of my curve and find very much larger values for the size of the wound than those given by the actual figures. As the geometrical progression has been found to hold in all the examples given with very considerable accuracy, I am inclined to think that du Noüy's interpretation is wrong and that the sepsis did make a permanent difference in the rate of healing.

APPENDIX.

Let it be assumed first that the wound be circular and that the rate of advance of the epithelium in the direction of the radius diminishes in a geometrical progression.

If the radius at any time be r, and A is the area, since

$$A = \pi r^2$$

we have at once

$$\frac{dA}{dt} = 2\pi r \frac{dr}{dt}$$

in which t is the time. But it has been assumed that $\frac{dr}{dt}$ diminishes in a geometrical progression so that

$$\frac{dr}{dt} = -ake^{-kt}$$

in which a is the radius at commencement of healing, whence

$$r = -ak\int e^{-kt}dt$$
$$= ae^{-kt}$$

so that we have

$$\frac{dA}{dt} = -2\pi a^2 e^{-2k}$$

or

$$A = \pi a^2 e^{-2k}$$

so that the area also decreases in a geometrical progression. This applies to a circular wound. In many of the experiments, the wound has been rectangular. Consequently, at the beginning of healing, the area covered by the advancing edge is rather greater than it would be were the edge circular, because the peripheral border of a square is greater in relation to the contained area than in the case of a circle, but the difference is not sufficient to interfere seriously with the analysis given above.

THE REVERSE SELECTIVE BACTERIOSTATIC ACTION OF ACID FUCHSIN.

By VICTOR BURKE, Ph.D., and CHARLES E. SKINNER.

(*From the Laboratory of Bacteriology of the State College of Washington, Pullman.*)

(Received for publication, September 27, 1923.)

Recent experiments upon the effects of dyes on cells have raised some interesting questions as regards the use of dyes in bacteriological technique and therapy. There is evidence to show that the majority of Gram-positive bacteria, *both vegetative cells and spores*, are more susceptible to the bactericidal action of gentian violet and other triphenylmethane dyes *than the vegetative cells of many* Gram-negative bacteria.[1,2,3] This selective action of gentian violet is known as selective bacteriostasis.[2] It has been utilized in inhibiting the growth of Gram-positive bacteria in methods of isolating or determining the presence of Gram-negative bacteria. It also delimits the value of these dyes in therapy.[2] Gentian violet has been utilized to check the growth of bacteria in wounds and on infected surfaces. It has not proven very effective in controlling the growth of Gram-negative organisms like *Bacterium coli* and *Pseudomonas pyocyanea*. Recent work suggests that certain dyes, or other substances, such as acid fuchsin, flavines, and sulfanilic acid, can be utilized to destroy the Gram-negative bacteria without affecting the Gram-positive spore bearers, and thus serve as an aid in the isolation of the latter.[3] The selective action of acid fuchsin has been termed reverse selective bacteriostasis. Furthermore, there is the possibility of combining with gentian violet some dye that will destroy the Gram-negative bacteria which are not affected by the gentian violet, thus making possible the use of a disinfectant effective against both types of bacteria.

[1] Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221.

[2] Churchman, J. W., *J. Am. Med. Assn.*, 1922, lxxix, 1657.

[3] Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 1.

The experimental work described in the present paper has been designed, first, to determine the value of acid fuchsin as a disinfectant and the desirability of combining acid fuchsin or some other dye showing reverse selective bacteriostasis with gentian violet for use in the treatment of mixed infections; and, second, to determine the cause of the reverse selective bacteriostasis and of the lack of parallelism between the bactericidal and bacteriostatic properties of acid fuchsin. An examination of the results indicates that acid fuchsin is a very weak disinfectant compared with some of the other dyes, especially in the presence of organic matter and at body temperature. This fact is so evident that little space will be devoted to it, the main discussion being confined to the reverse selective action of acid fuchsin and the lack of parallelism between the bactericidal and bacteriostatic properties.

Reverse Selective Bacteriostatic Action of Acid Fuchsin.

The Gram-positive non-spore bearers are not included in the selective action of acid fuchsin and nothing is known about the resistance of the Gram-negative spore bearers. It is thus seen that the reversed selective action is only partial, certain groups apparently being exempt. The fact that the reversed action applies only to the Gram-positive spore bearers naturally suggests that the apparent reversal is due to spore resistance. Gentian violet destroys both vegetative cells and spores of the Gram-positive bacteria in weaker dilution than it does most of the Gram-negative bacteria.[3] Does acid fuchsin spare both the vegetative cells and spores of the Gram-positive bacteria in weaker dilution than it does the Gram-negative bacteria? We have the resistance of three elements to consider, the Gram-negative vegetative cells, the Gram-positive vegetative cells, and the Gram-positive spores. If only the spores resist the acid fuchsin then we fail to see any significance in the reversed selective action of acid fuchsin. Furthermore, if the Gram-negative bacteria resist the bacteriostatic action of acid fuchsin better than the vegetative cells of Gram-positive spore bearers we have an explanation of the lack of parallelism between the bactericidal and bacteriostatic action of this dye.

For a complete analysis and comparison of the gentian violet reaction with the acid fuchsin reaction it is necessary to determine separately the resistance of vegetative cells and spores of the Gram-positive bacteria to acid fuchsin. A study of the Gram-negative spore-bearing organisms is desirable but not essential. For the acid fuchsin reaction to be comparable with the gentian violet reaction but the reverse thereof, it must be demonstrated that the Gram-positive vegetative cells, freed of spores, resist acid fuchsin in higher dilution than do the Gram-negative vegetative cells. The following experiments demonstrate the comparative resistance of Gram-negative bacteria and the vegetative cells and spores of Gram-positive bacteria.

TABLE I.

Results of an Experiment to Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore-Bearing Organism in the Presence of Organic Matter and at a Temperature of 45°C.

Organism.	Treatment.	Organisms surviving per cc.
B. subtilis (1).	4 cc. of culture mixed with 1 cc. of saturated aqueous solution of acid fuchsin heated to 45°C. for 1 hr.*	690,000
" " (2).	Control; the same as No. 1 except 1 cc. of broth added in place of acid fuchsin.	48,000,000
" " (3).	The same as No. 2 except heated to 75°C. for 15 min.	720,000

* To make a saturated solution 4 or 5 gm. of the dye were added to 10 cc. of distilled water. Coleman and Bell Co. recommended adding 10 gm. of their dye to 10 cc. of water to get a saturated solution. In most of our experiments the National Aniline and Chemical Company's acid fuchsin was used. This company advises us that their acid fuchsin is soluble to the extent of 4 to 5 per cent.

Experiment 1. To Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore-Bearing Organism in the Presence of Organic Matter and at a Temperature of 45°C.—1 cc. of a saturated aqueous solution of acid fuchsin (Coleman and Bell Co.) was added to 4 cc. of a calcium carbonate dextrose broth culture of *B. subtilis*. The culture of *B. subtilis* was obtained by mixing a 24 hour culture and a 7 day culture. Spores were present. The mixture was heated for 1 hour at 45°C. plated out quantitatively and then incubated at 37°C. for 24 hours. Counts were made in the usual manner. In order to determine whether the vegetative cells as well as spores survived, 4 cc. of the culture were mixed with 1 cc. of broth and heated to 75° C. for 15 minutes. The control consisted of 4 cc. of the culture mixed with 1 cc. of broth and heated to 45° C. for 1 hour.

The results obtained are given in Table I. These indicate that under the conditions of the experiment acid fuchsin destroys the vegetative cells of *Bacillus subtilis* but not the spores.

The presence of organic matter reduces the action of acid fuchsin as well as that of most other dyes except the flavines and favors the resistance of the vegetative cells. It should be noted that in this experiment organic matter was present and yet the vegetative cells failed to survive the exposure to acid fuchsin. The experiment demonstrates that the vegetative cells and spores of a Gram-positive spore bearer differ in their resistance to acid fuchsin. This fact should be taken into consideration in any discussion of reverse selective bacteriostasis.

The experiment was repeated using sulfanilic acid 1 cc. to 1 cc. of the broth culture with comparable results. The vegetative cells were killed and the spores survived.

Experiment 2. To Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore Bearer in the Absence of Organic Matter.—Transplants of *B. subtilis* were made on agar slants at 12 hour intervals for 48 hours, and the growth on the fourth transplant was washed off in saline. This reduced the spores to a minimum. The other conditions of the experiment were the same as those described in Experiment 1.

The results obtained are given in table II and indicate that under the conditions of the experiment acid fuchsin destroys the vegetative cells of *B. subtilis* while the spores survive.

Experiments 1 and 2 suggest that the reverse selective action attributed to acid fuchsin applies only to the spores and not to the vegetative cells of the Gram-positive spore bearers. In broth and saline suspension the spores resist high concentration of acid fuchsin while the vegetative cells are killed. The results of Experiment 3 indicate that *Bacterium coli* in broth is very resistant to acid fuchsin.

Experiment 3. To Determine the Effect of Acid Fuchsin on Bacterium coli in Dilutions Destroying the Vegetative Cells of Gram-Positive Spore Bearers.—The technique of the experiment was the same as in Experiments 1 and 2. Comparison is also made with a Gram-positive non-spore bearer. 24 hour calcium carbonate broth cultures and saline suspensions were used (Table III).

The experiment was repeated, using *Bacterium cloacæ* and *Sarcina lutea* with essentially the same results.

Bacterium coli resisted acid fuchsin in dilutions destroying the vegetative cells of *Bacillus subtilis*. It was destroyed in saline suspension in dilutions of acid fuchin not destroying the spores of *Bacillus subtilis*. *Bacterium coli* appears to be fully as resistant,

TABLE II.

Result of an Experiment to Determine the Effect of a Strong Solution of Acid Fuchsin on a Gram-Positive Spore Bearer in the Absence of Organic Matter.

Organism.	Treatment.	Organisms surviving per cc.
B. subtilis (1).	4 cc. of suspension mixed with 1 cc. of saturated aqueous acid fuchsin and heated to 45°C. for 1 hr.	50
" " (2).	Control; the same as No. 1 except 1 cc. of saline added in place of acid fuchsin.	23,000,000,000
" " (3).	The same as No. 2 except heated to 75°C. for 15 min.	53

TABLE III.

Result of an Experiment to Determine the Effect of Acid Fuchsin on Bacterium coli in Dilutions Destroying the Vegetative Cells of a Gram-Positive Spore Bearer.

Organism.	Treatment.	Organisms surviving per cc.
Bacterium coli (1).	4 cc. of broth culture mixed with 1 cc. of saturated aqueous acid fuchsin heated to 45°C. for 1 hr.	130
" " (2).	Control; the same as No. 1 except 1 cc. broth used in place of acid fuchsin.	130,000,000
" " (3).	The same as No. 1 except saline suspension organisms used.	None.
" " (4).	Same as No. 3 except 1 cc. of saline used in place of 1 cc. of acid fuchsin.	3,800,000
Staphylococcus albus (5).	Same as No. 1.	680
" " (6).	" " " 2.	88,000,000
" " (7).	" " " 3.	None.
" " (8).	" " " 4.	2,800,000

if not more resistant, to acid fuchsin than the vegetative cells of the Gram-positive spore bearers. This refers to the bactericidal effect. Later experiments demonstrate that the Gram-negative bacteria such as *Bacterium coli* are more resistant than the Gram-

positive spore bearers to the bacteriostatic action of acid fuchsin. And the flavines which are credited with the same properties as acid fuchsin have greater bacteriostatic action on the Gram-positive non-spore bearers than on the Gram-negative bacteria. It follows that the reverse selective action of acid fuchsin indicates only spore resistance.

The results given in Table III indicate variation in the resistance of the individual cells of both species used, 130 *Bacterium coli* cells out of 130,000,000, and 680 *Staphylococcus albus* cells out of 88,000,000 surviving. Whether this variation in resistance to acid fuchsin can be utilized to separate the species into resistant and non-resistant strains to acid fuchsin as done by Churchman with *Bacterium coli* and gentian violet has not been determined. The individual cells of any species of bacteria manifest differing amounts of resistance when exposed to unfavorable conditions. This is well known to be true also of protozoan parasites. Whether the variation in resistance shown in Table III represents simple individual variation, strain variation, or the development of a resistant strain remains to be determined.

As bearing on the general question of reverse selective action it seemed advisable to determine the effect of acid fuchsin on a Gram-negative spore bearer. *Bacillus brevis* was used and the conditions of the experiment were the same as those given in Table II for *Bacillus subtilis*, except that older cultures were used. The results are given in Table IV. Under the conditions of this experiment the vegetative cells of *Bacillus brevis* are killed and the spores survive. The Gram-positive and Gram-negative spore bearers under the conditions of the experiment were affected in the same way by acid fuchsin. By varying the concentration of the dye the results might conceivably have been modified but no experiments of the sort were made. The effect of gentian violet on Gram-negative spore bearers is unknown to us.

We were unable to obtain cultures of *Bacillus subtilis* free of spores. 12 hour transplants were found to contain spores. However, our 24 hour cultures of *Bacillus brevis* were spore-free though spores were present in old cultures. Exposing 24 hour cultures of *Bacillus brevis* to the acid fuchsin was found to destroy all the cells. If the

experiments had been discontinued at this point and the results compared with those obtained with *Bacillus subtilis* the conclusion might have been reached that the reverse selective bactericidal action attributed to acid fuchsin should be extended to include the Gram-negative spore bearers. But the investigation of the effect of acid fuchsin on the spores of *Bacillus brevis* demonstrated that reverse selective action does not apply to the Gram-negative spore bearers.

TABLE IV.

Result of an Experiment to Determine the Effect of Acid Fuchsin on a Gram-Positive and a Gram-Negative Spore Bearer.

Organism.	Age of culture.	Treatment.	Organisms surviving per cc.
B. subtilis (1).	18 hrs. and 30 days.	4 cc. of suspension mixed with 1 cc. of saturated aqueous acid fuchsin, heated 1 hr. at 45°C.	23,000
" " (2).	18 hrs. and 30 days.	Control; 1 cc. of saline used in place of acid fuchsin, treated as above.	8,700,000,000
" " (3).	18 hrs. and 30 days.	The same as No. 2 except exposed to 75° C. for 15 min.	29,000
" *brevis* (4).	18 hrs. and 30 days.	Same as No. 1.	2,300
" " (5).	18 hrs. and 30 days.	" " " 2.	3,700,000
" " (6).	18 hrs. and 30 days.	" " " 3.	1,900
" *subtilis* (7).	24 hrs.	" " " 1.	180
" " (8).	24 "	" " " 2.	59,000,000,000
" " (9).	24 "	" " " 3.	220
" *brevis* (10).	24 "	" " " 1.	None.
" " (11).	24 "	" " " 2.	180,000,000
" " (12).	24 "	" " " 3.	None.

Acid fuchsin is but a weak germicide. Spores of both Gram-negative and Gram-positive bacteria resist it in greater concentration than do the vegetative cells. It follows that the selective action of acid fuchsin is not comparable with the selective action of gentian violet.

Experiment 4. To Compare the Effect of Acid Fuchsin on a Gram-Positive and a Gram-Negative Spore Bearer.—The technique of the experiment was the same as that of Experiment 2. Saline suspensions of the organism were used. To insure

cultures containing both spores and vegetative cells 18 hour and 30 day cultures were mixed. 24 hour cultures were also used to insure either a small number of spores or a lack of spores.

In order to determine whether enough acid fuchsin was being carried over to the plates to inhibit growth, sufficient saturated acid fuchsin was added to agar to make a 1:100 saturated solution. This is a more concentrated solution than was present in any of our plates. *Bacterium coli*, *Bacterium cloacæ*, *Staphylococcus albus*, *Sarcina lutea*, *Bacillus brevis*, and *Bacillus subtilis* grew as well on this agar as in agar without the dye, a fact which proves that the action of acid fuchsin in our experiments was bactericidal rather than bacteriostatic. Molds grow readily on the surface of saturated aqueous acid fuchsin.

It has been suggested that the reverse selective action of acid fuchsin may be due to the presence of the SO_3 groups,[3] acid fuchsin being originally produced by the process of sulfonation from basic fuchsin, which has the same selective action as gentian violet. Churchman states that "addition of the sulfonic acid groups not only makes the basic triphenylmethanes more toxic for Gram-negatives but less toxic for Gram-positive spore-bearing aerobes." But if the reverse selective action of acid fuchsin is purely a question of spore resistance, as our experiments indicate, one would expect to find that the process of sulfonation makes the basic fuchsin less toxic for the Gram-negative bacteria as well as for the Gram-positive organisms. In other words, the altered dye should act as an ordinary germicide and a reduction in its action on the Gram-positive spore bearers would be accompanied by a reduction in its action on Gram-negative bacteria.

Experiment. 5. To Determine the Effect of the Addition of SO_3 Groups on the Bactericidal Action of Basic Fuchsin for a Gram-Negative Organism.—In addition to basic fuchsin and acid fuchsin other dyes were used to determine comparative bactericidal powers.

Technique of the experiment: To 4 cc. of a saline suspension of *Bacterium coli* was added 1 cc. of a solution of the dye. The dyes were made in different solutions as given in the table. The tubes were exposed to a temperature of 45°C. At intervals up to 5 hours strokes were made on agar to determine viability. The dye carried over did not produce bacteriostasis.

The results are given in Table V. It is seen that basic fuchsin is a more powerful disinfectant than acid fuchsin for the Gram-negative bacteria as well as for the Gram-positive bacteria. The addition of the SO_3 group to basic fuchsin has decreased the bactericidal action for the Gram-negative as well as the Gram-positive bacteria. The process of sulfonation has reduced or eliminated the specific action characteristic of the basic triphenylmethane dyes for the spores of the Gram-positive bacteria. The staining power is also reduced.

TABLE V.

Results of an Experiment to Determine the Effect of the Addition of SO_3 Groups on the Bactericidal Action of Basic Fuchsin for the Gram-Negative Organism Bacterium coli.

Min.....	0	1	5	10	15	30	60	90	180	300
Basic fuchsin, 1 gm. in 100 cc. of H_2O.....	+++*	+++	+++	+++	++	++	+	+	+	0
Acid fuchsin, 3 gm. in 10 cc. of H_2O.....	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Crystal violet, 0.5 gm. in 100 cc. of H_2O	+++	+++	0	0	0	0	0	0	0	0
Mercurochrome 220, 0.5 gm. in 100 cc. of H_2O.....	+++	+++	+++	+++	+++	++	++	+	+	+
Neutral acriflavine, 0.5 gm. in 100 cc. of H_2O.....	+++	+++	++	++	++	+	0	0	0	0

* +++ indicates as much growth as was shown by the control.

An examination of Table V indicates that under the conditions of this experiment crystal violet is more bactericidal for *Bacterium coli* than neutral acriflavine. We should not expect this to hold true in infected tissues since other experimental work has shown that in serum the bactericidal action of basic triphenylmethane dyes is decreased and that of the flavines increased.

DISCUSSION.

Bactericidal Action of Acid Fuchsin.

Our experiments show that acid fuchsin is not a strong disinfectant, particularly in the presence of organic matter. Churchman's experiments with acid fuchsin were made by combining 5 drops of a saturated aqueous solution of acid fuchsin to 20 drops of an aqueous suspension of bacteria, held at 45°C. for 1 hour, then stroking on agar to determine viability.[3] He expresses the opinion that a weaker solution of the dye would have been effective. It should be noted that 20 per cent of a saturated solution represented a great concentration of the dye, that the experiments were conducted in the absence of organic matter and in the presence of heat. Beckwith has shown that a dilution of less than 1:1,000 of acid fuchsin at 37°C. will kill *Bacterium typhosum* in bouillon in 24 hours;[4] also that the action of the dye increases with an increase in acidity. But it is not advisable to increase the acidity of body fluids in most cases of infection.

Our experiments would seem to indicate that acid fuchsin can have little or no value as a disinfectant in the treatment of wounds, for it is, as just mentioned, a very weak disinfectant in the presence of organic matter. The possibility of combining it with gentian violet or other triphenylmethane dyes for the purpose of inhibiting the Gram-negative bacteria does not appear to be an important one. It is true that the Gram-negative bacteria, such as *Bacterium coli* and *Pseudomonas pyocyanea*, are difficult to eliminate from wounds with gentian violet. However, gentian violet is toxic to these organisms in greater dilution than is acid fuchsin. Apparently nothing would be gained by adding acid fuchsin.

It is doubtful if acid fuchsin can be utilized in the separation of bacterial species to the same extent as gentian violet. The use of heat is a more practical and cheaper method of isolating spore bearers. Neither heat nor acid fuchsin can be used if spores are not present. These statements apply to the bacteria as a group. It is, of course, conceivable that under certain conditions the use of

[4] Beckwith, T. D., *J. Infect. Dis.*, 1921, xxix, 495.

acid fuchsin may serve to separate some Gram-positive bacteria from Gram-negative ones. But it cannot be used in isolating Gram-positive spore bearers directly from wounds since bacteria rarely produce spores in the body.

Bacteriostatic and Bactericidal Power of the Dyes.

A chemical substance may stimulate the growth of bacteria in one strength, inhibit the growth of bacteria in greater concentration, and kill bacteria in still greater concentration. Certain substances may serve very well as inhibiting or bacteriostatic agents but prove to be of little value as bactericidal agents. In general it has been assumed that bacteriostatic and bactericidal properties run parallel; *i.e.*, any substance that will inhibit the growth of one bacillus in greater dilution than it will inhibit the growth of a second bacillus will also kill the first bacillus in greater dilution than it will kill the second bacillus. This refers to vegetative cells. The presence of spores introduces a separate factor. It is evident that a substance may inhibit a spore-bearing organism in weaker dilution than a non-spore-bearing organism but on account of the extreme resistance of spores not kill the spore-bearing organism in any dilution while killing the non-spore-bearing organism. In such a case it might be stated that the bacteriostatic and bactericidal properties do not run parallel.

Recently attention has been drawn to a lack of parallelism between the bacteriostatic and bactericidal properties of acid fuchsin.[3,5] Our experiments indicate that this lack of parallelism is due to spore resistance.

The suggestion made by Churchman that some dye specific for the Gram-negative bacteria might be combined with gentian violet for the preparation of a disinfectant destructive to both groups of bacteria is worthy of consideration. However, every dye so far investigated that will destroy the Gram-negative bacteria such as *Bacterium coli* and *Pseudomonas pyocyanea* in tissues has been found also to destroy or inhibit the Gram-positive bacteria exclusive of the spores. From the evidence of our experiments (Table V) it

[5] Churchman, J. W., *J. Exp. Med.*, 1923, xxxvii, 543.

seems probable that the SO_3 group will have little value in comparison with the triphenylmethane or flavine dyes in the treatment of infections with either Gram-positive or Gram-negative bacteria. We are investigating the possibility of combining two dyes of different molecular structure without reducing the bactericidal action of either.

Since most of the spore bearers rarely if ever produce spores in the body and since, furthermore, the pathogenic spore bearers are mainly Gram-positive and susceptible to the triphenylmethane dyes, spore resistance is not a weighty clinical problem. It is true that infection often arises from spores introduced into fresh wounds but after the initial stage of infection they cease to be a factor save when they remain dormant and constitute a menace for the future.

CONCLUSIONS.

1. The reverse selective action attributed to acid fuchsin can be explained on the basis of spore resistance. This action does not apply to the vegetative cells of Gram-positive spore bearers. It cannot be extended to include the Gram-negative spore bearers.
2. The selective action of acid fuchsin is that of an ordinary weak disinfectant and is not comparable to the selective action of the triphenylmethane dyes.
3. A lack of parallelism between the bactericidal and bacteriostatic action of acid fuchsin can be explained on the basis of spore resistance and it does not exist when spores are excluded.
4. Acid fuchsin is a weaker disinfectant than basic fuchsin for both Gram-negative and Gram-positive bacteria. The process of sulfonation by which acid fuchsin is made from basic fuchsin results in a reduction in the bactericidal action of the dye for Gram-negative and Gram-positive bacteria.
5. The most significant fact in the action of the dyes on bacteria is the toxic effect of the triphenylmethane dyes on Gram-positive spore-bearing organisms.